University of Alberta

# Establishing a Framework for Mapping DQ8 Restricted T cell Epitopes for Human Diabetes Autoantigens

by

Lisa Purdy

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **ESTABLISHING A FRAMEWORK FOR MAPPING DQ8 RESTRICTED T CELL EPITOPES FOR HUMAN DIABETES AUTOANTIGENS** submitted by **LISA EILEEN PURDY** in partial fulfillment of the requirements for the degree of **MASTER OF SCIENCE.**

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## DEDICATION

This thesis is dedicated to the three most important people in my life. First to my parents, thank you for your support through 21 years of education. Just knowing how proud you are of me no matter which road I journey down has enabled me to tackle anything. And to Rod, I believe your constant love and understanding has empowered me to continue on when the waters get rough. Thank you for listening and holding my hand.

## ABSTRACT

Type I diabetes susceptibility has been linked to the DQ8 allele of HLA, which is present in 90% of diabetics. The autoimmune process in diabetes appears to be primarily mediated by T lymphocytes as several islet cell antigens have been shown to be recognized by CD4 T cells from diabetics. This thesis describes a general approach to begin elucidating the DQ8 restricted T cell epitopes within diabetes autoantigens such as human proinsulin and glutamic acid decarboxylase. A new lineage of mice that express only human DQ8 class II molecules in the absence of mouse class II was established. Lymph node cells from the DQ8 $IA\beta^{null}$ mice mounted an immune response that could be blocked by monoclonal antibodies to human class II and splenocytes produced IL-2 when stimulated with superantigens. This proves that DQ8 is responsible for positive and negative selection in the thymus of these mice. In order to map diabetes autoantigens a new vector to transfect into antigen presenting cells was created.

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## ABBREVIATIONS

| | |
|---|---|
| **2-ME** | 2-mercaptoethanol |
| **AA** | amino acid |
| **APC** | antigen presenting cell |
| **Asp** | asparagine |
| **Arg** | arginine |
| **bp** | base pairs |
| **CDR** | complementary determining region |
| **CFA** | complete freund's adjuvant |
| **CLIP** | class II associated invariant chain peptide |
| **Cntrl** | control |
| **Con A** | concanavalin A |
| **CPM** | count per minute |
| **CMV** | cytomegalovirus |
| **DNA** | deoxyribonucleic acid |
| **DEAE** | diethylaminoethyl |
| **DEPC** | diethylpyrocarbonate |
| **DME** | Dulbecco's modified eagle's medium |
| **DMSO** | dimethyl sulfoxide |
| **ds** | double stranded |
| **EAE** | experimental allergic encephalomyelitis |
| **EBNA** | Epstein Barr nuclear antigen |
| **EDTA** | ethylene-diamine tetra acetic acid |
| **EF1α** | human elongation factor 1 alpha |
| **ER** | endoplasmic reticulum |
| **FACS** | fluorescence activated cell sorting |
| **FCS** | fetal calf serum |
| **FITC** | fluorescent isothiocyanate |
| **GAD** | human glutamic acid decarboxylase (65kDa isoform) |
| **GFP** | green fluorescent protein |
| **HLA** | human leukocyte antigen |
| **hph** | hygromycin phosphotransferase gene |
| **Ii** | human invariant chain p31 isoform |
| **Iono** | ionomycin |
| **Kb** | kilo bases |
| **LN** | lymph node |

| | |
|---|---|
| **mAmps** | milliamperes |
| **MHC** | major histocompatibility complex |
| **MBP** | myelin basic protein |
| **NK** | natural killer (cells) |
| **NOD/Lt** | non obese diabetic mice from the Leiter colony (Jackson Labs) |
| **OVA** | chicken ovalbumin |
| **ORF** | open reading frame |
| **PBL** | peripheral blood lymphocytes |
| **PBS** | phosphate buffered saline |
| **PE** | phycoerytherin |
| **PMA** | phorbol mystic acid |
| **RFLP** | restriction fragment length polymorphisms |
| **sAG** | superantigen |
| **SEA** | staphylococcal enterotoxin A |
| **SEB** | staphylococcal entertoxin B |
| **Ser** | serine |
| **SI** | stimulation index |
| **SIg** | surface immunoglobulin |
| **SV 40** | simian virus 40 |
| **TE** | tris EDTA buffer |
| **Tg** | transgenic |
| **TSST-1** | toxic shock syndrome toxin-1 |

# CHAPTER I

## GENERAL INTRODUCTION

The immune system has evolved to become our important defense mechanism against invading pathogenic microorganisms and cancer. Many cells and molecules in the immune system function in tandem to recognize and rid the host of foreign assailants. Periodically the rigid control of the immune response fails and self-tolerance is broken. The result is what is termed autoimmunity.

### Cellular versus Humoral Immunity

There are two branches of the immune system, cellular immunity and humoral immunity. There is a cooperative interaction between the two in order to initiate an appropriate immune response; however they do have different primary functions. The humoral arm of the immune system involves the interactions between B cells and antigen and results in B cell proliferation and differentiation into immunoglobulin-producing plasma cells, whereas the cellular arm is involved more with T cells and natural killer (NK) cells that destroy tumor cells or cells harboring viruses. Most of the recognition molecules of the immune system belong to the immunoglobulin superfamily (1). This includes immunoglobulins that recognize native foreign antigens as well as the T-cell receptor (TCR) (2) used by T cells to recognize processed foreign antigenic peptides that are presented on the surface of antigen presenting cells (APCs) in the context of MHC class I (recognized by CD8+ T cells) or MHC Class II (recognized by CD4+ T cells).

### Genes of the MHC Complex

The major histocompatibility complex is a 4000 kb cluster of genes located on the

short arm of chromosome 6 in humans (HLA complex) and on chromosome 17 in mice (H-2 complex). The structure of this region has been determined through RFLP studies, deletion mutant studies, cosmid walking, and pulse-filed gel electrophoresis (3,4). The region encodes three groups of molecules: class I on the telomeric end , class II on the centromeric end, and class III in the middle (figure 1.1, at the end of this chapter). Class I genes encode for glycoproteins found on the surface of essentially all nucleated cells, class II genes encode for the glycoproteins expressed on APCs, and class III genes encode proteins associated with the immune system, for example complement and tumor necrosis factor. The work in this thesis pertains to MHC class II. This molecule will be discussed in more detail below.

**HLA Class II Genes**

The MHC class II region contains genes encoding for α and β polypeptide chains of three different molecules; HLA-DR, DP, and DQ. In some cases there is more than one functional gene for each molecule -- for example the DR subregion contains up to four expressed DRβ genes (5) (figure 1.1). All of these DRβ genes can be expressed in combinations with the nonpolymorphic DRα, thereby increasing the number of different class II molecules expressed on the cell surface. In contrast to the DR molecules, only one of DQα (DQA1) and DQβ (DQB1) is expressed. Both DQA1 and DQB1 show allelic polymorphism with at least 16 variants of DQA1 and 25 variants of DQB1 discovered thus far (6,7). HLA-DP also consist of two pairs of genes; however, only the DPα1 and DPβ1 are expressed. The allelic polymorphism in the HLA genes means that a vast number of different foreign peptides can be presented on class II molecules, and therefore, in theory no matter what pathogen is encountered a subset of the population will be able to mount an immune response against it. In general the DQα protein encoded for on one chromosome can pair with the DQβ from either the same chromosome (cis) or the opposite chromosome (trans) so that four different DQ heterodimers can be formed. This

phenomenon is also seen with DP. The large number of different class II molecules (up to eight different DRs, four different DQs, and four different DPs) expressed in any given individual contributes to the large number of different peptides that can be recognized in order to mount an effective CD4 T cell immune response.

The nomenclature for class II is notorious for its confusion. In this thesis when referring to HLA molecules the following nomenclature will be used: e.g. DQA1*0301 is the α chain of DQ, the specific allele is 0301.

**Linkage Disequilibrium**

One important feature of the HLA complex is linkage disequilibrium (two alleles are inherited together with a abnormally high frequency). Even though the distance between DQβ1 and DRβ1 is approximately 175 kb (great enough for recombination to occur) these genes are generally inherited as a single 'unit' together with DQα1 (8). Todd speculates that the disequilibrium is probably due to natural selection, as coordination of the MHC expression would have functional advantages during thymic selection of the T-cell repertoire and/or be advantageous in conferring resistance to infectious diseases (9,10). The problem with linkage disequilibrium comes when scientists are trying to map disease susceptibility loci, since when two genes are tightly linked it is not immediately obvious which of the two is primarily responsible for the phenotype.

There are three other types of genes located in the class II region. The first are genes encoding proteasome subunits, LMP 2 and LMP 7, thought to be involved in the complex that mediates cytoplasmic degradation of endogenous proteins into peptides. TAP1 and TAP2 encode peptide-transport subunits involved in the transfer of peptides into the lumen of the ER so they can interact with class I molecules. The HLA-DM α and β genes are also located in this region and will be discussed below.

The murine class II locus is located on chromosome 17 and contains two pairs of genes -- I-E α/β and I-A α/β. I-E most resembles the HLA-DR, with only the β genes

being polymorphic, while I-A is an analog of HLA-DQ; both the α and β genes are polymorphic.

**Expression of MHC class II**

The α genes have five exons, encoding respectively for the signal sequence, the α1 domain, the α2 domain, the transmembrane region including the cytoplasmic tail, and the 3' untranslated region (UT). The β genes are essentially the same except the 3'UT region is located on exon six. When transcribed and translated the MHC class II genes encode for a 33 kDa α chain and a 28 kDa β chain that are associated by noncovalent interactions. The combined chains form a membrane-distal peptide binding domain (which is polymorphic to allow for binding to a great variety of different peptides), a membrane proximal domain (consisting of two immunoglobulin domains), a transmembrane segment, and a cytoplasmic anchor segment. The membrane proximal and subsequent domains are highly conserved, and hence the overall structure of the class II molecule is relatively conserved (11).

**MHC Class II Structure**

The elucidated crystal structure of DR1 demonstrates that a peptide binding groove is formed by the α1 of β1 domains which generate both the floor (eight anti-parallel β strands) as well as the side walls (two anti-parallel α helices) of the groove (11). The peptide binding groove is open at both ends so the peptide can extend beyond the class II molecule, allowing for larger peptides (up to 18 aa) to be presented. In the peptide binding groove there are side pockets which interact with particular residues of the peptide. The specific region of the peptide that binds to the pockets is typically 7-10 aa long and depending on the MHC molecule, the bound region of the peptide generally has aromatic or hydrophobic residues at the N terminus and three additional hydrophobic

residues in the middle or C terminus. These anchor the peptide by fitting into the pockets of the groove.

**Antigenic Processing**

Peptides find their way onto the class II molecule through two different mechanisms. The first is simple exchange on the cell surface of one peptide from the binding groove for another from the environment. The second mechanism involves a more complex pathway whereby proteins are internalized, digested, and processed so that at least a subset of the resulting peptides is associated with class II (in the peptide binding groove). The assembled class II plus peptide is then transported to the cell surface. The APC internalizes the antigen by either phagocytosis (macrophages or dendritic cells) or endocytosis (macrophages or B cells). The exogenous antigen is degraded into peptides as it moves through the early endosomal, late endosomal, and lysosomal compartments. In each compartment the pH decreases and hydrolytic enzymes such as proteases, nucleases, glycosidases, lipases, phospholipases, and phosphatases become more abundant. The α and β chains of each class II molecule are able to pair efficiently and remain stably associated in the ER due to the interaction with invariant chain (Ii) (12-14). Three class II heterodimers associate with each Ii trimer (15). Each Ii monomer interacts with the peptide binding groove of a single α/β heterodimer via the CLIP region (class II-associated invariant chain peptide) (residues 83-107) (16-18), both stabilizing the heterodimer and preventing endogenous peptides from binding (19,20). Ii contains signal sequences (residues 1-19) (21-23) that are involved with the intracellular transport of the class II molecules out of the ER, through the Golgi complex and into the endosomal compartment. Essentially the α/β class II heterodimer is unstable and assembles poorly unless it has a peptide to 'condense around' to fill the peptide binding groove, and in the ER the CLIP region of Ii provides the necessary peptide. The class II molecules associated with Ii take the same route as the protein being processed; that is early to late

endosomal compartments and finally to the lysosome. As the proteolytic activity increases along the way, the Ii is degraded. However because it is protected from degradation by its location within the peptide binding groove, the CLIP remains intact and prevents premature peptide binding. The class II / CLIP complex finally ends up in the same endosomal / lysosomal vesicles that contain exogenously derived antigenic peptides. Because of the acid environment, the class II confirmation is changed to a more open form termed 'floppy'. Also located in this compartment is HLA-DM, a molecule encoded for in the class II region that consists of $\alpha$ and $\beta$ chains that associate to form a heterodimer like classical class II molecules (24). The function of HLA-DM is to mediate the removal of CLIP from the class II binding groove so that other potentially antigenic peptides can be loaded (24,25). This is facilitated by the 'floppy' confirmation of the class II molecule. After binding to a peptide which gives an acid stable complex, the resulting $\alpha/\beta$ heterodimer plus peptide moves to the cell surface to possibly interact via the T cell receptor (TCR) with CD4 T cells.

**The T Cell Receptor**

The TCR is found on the surface of T cells. It recognizes peptides that are presented on class I (in the case of CD8+ T cells ) or class II (in the case of CD4+ T cells). The TCR is a heterodimer of $\alpha$ and $\beta$ (90-99% of T cells) or $\gamma$ and $\delta$ chains that closely resemble other members of the immunoglobulin superfamily (26). Each chain in the TCR has two domains; the N-terminal variable domain (V) which has three hypervariable regions (which represent three complementary determining regions; CDR1, CDR2, and CDR3) and a constant (C) domain which is conserved (figure 1.1). Each TCR chain also has a short connecting sequence that contains a cysteine residue to link the two chains of the TCR complex together. The carboxy-terminus of the chains is composed of two regions; a positively charged transmembrane region that anchors the TCR into the cell membrane and allows it to interact with the signal-transducing CD3 complex and a short

cytoplasmic tail (25). The α chain is encoded by the V, J, and C gene segments, while the β chain is encoded for by V, D, J, and C gene segments. Rearrangement results in V J joining for the α chain and V D J joining for the β chain. The diversity of the TCR is accomplished through these gene rearrangements as there are thousands of possible combinations between V J and V D J (2). The genetic rearrangement of the TCR occurs in the thymus during T cell maturation. During this process T cells are both positively and negatively selected. Positive selection involves the interaction of immature T cells with the epithelial cells of the cortex of the thymus (27-29). Only those T cells expressing TCRs capable of binding to self MHC complex survive, ensuring that they have some affinity for self MHC. Negative selection occurs in the medulla of the thymus and is the result of the deletion of the TCR binding strongly to the MHC of dendritic cells and macrophages (30). This results in tolerance to self antigens as T cells that are self-reactive are destroyed.

**Antigen Presentation and T Cell Activation**

During an immune response the interaction between the TCR and MHC peptide complex is essential to the development of both humoral and cell-mediated immunity. The TCR recognizes the MHC peptide complex and binds to the top of this complex (figure 1.2) (31). The entire crystal structure of the co-complex has yet to be solved, making it impossible to be certain exactly how the MHC complex and TCR interact (32,33). It is currently thought that CDR1 and CDR2 are responsible for interacting with the MHC α1 and β1 domains (34), whereas the peptide binding region of the TCR appears to map largely to the CR3 (35).

The most important coreceptor in this interaction is CD4. CD4 binds to the β2 domain of class II (figure 1.2). This interaction increases the avidity between the TCR and peptide MHC complex. The CD4 cytoplasmic domain also plays a role in signal transduction, which ultimately is responsible for T cell activation. Activation of T cells includes transcription of interleukin-2 (IL-2) and IL-2 receptor, which allows for the T cell

to proliferate (36). There are other co-signaling molecules on the T cell that aid in the adhesion of the T cell to the APC. These include CD2, LFA-1, CD28, and CD45R. These accessory molecules are not directly involved with the TCR / MHC complex but rather bind to separate ligands on the APC; namely LFA-3, ICAM-1, B7, and CD22 respectively. CD28 is present on approximately 80% of peripheral T cells and the amount increases upon activation of the T cell. CD28 association with B7 on the APC is important for promoting the interaction between the T cell and APC as well as for signal transduction within the T cell. CD2 on the T cell associated with LFA-3 on the APC is also important in adhesion and signal transduction as classical experiments have shown that T cell responses are only significant when CD2 and LFA-3 interact (Immunology 452 class notes, 1994). LFA-1 on the T cell interacts with ICAM-1 on the APC and is also involved with signal delivery. LFA-1 is increased on the T cell after activation. The cytoskeleton of the T cell changes slightly upon the interaction of co-stimulatory molecules with the ligands on the APC. These interactions are vital for complete activation of the T cell by increasing the avidity of the interaction between the T cell and the APC.

**HLA and Disease**

Over time it has been observed that there is a close association between certain HLA alleles and susceptibility to various diseases (37). Examples of this include rheumatoid arthritis (37), celiac disease (38), pemphigus vulgaris (39), multiple sclerosis (40), and insulin-dependent diabetes mellitus (IDDM) (41), as well as over 70 other diseases (37).

**Insulin-Dependent Diabetes Mellitus**

IDDM is a disease that was and in some respects continues to be, a mystery to clinicians and scientists. At the turn of the century it was understood that the pancreas played a role in the pathophysiology of diabetes, but it was not until the early 1920's when Banting, Best and Collip first purified insulin and began using it therapeutically that this

was clarified (42). It is now understood that insulin is secreted by β cells which are located in the islets of Langerhans in the pancreas. Diabetes is a chronic autoimmune disease of these cells that is characterized by an infiltration of mononuclear cells into the islets, resulting in destruction of the β cells (43). Disease progression has been successfully blocked using cyclosporin A therapy, thus this is consistent with the view that CD4 T cells are important in orchestrating disease progression (44). The anti-islet autoantibodies which arise during β cell destruction are frequently present years before the first clinical signs of disease (45,46). Insulin is responsible for the maintenance of carbohydrate metabolism through transport of glucose into cells. Uncontrolled diabetes presents clinically as increased glucose levels in the blood and eventually in the urine (as the reabsorption threshold of the kidneys is surpassed). Modern monitoring of diabetic control is through the measurement of hemoglobin $A_{1c}$. This gives an integrated measure of glucose regulation over a period of approximately four months (the life span of a red blood cell). With persistent elevated blood glucose levels many proteins in the body become more highly glycosylated and this phenomenon is conveniently measured using the hemoglobin molecule.

With the advent of insulin therapy, acute deaths due to the metabolic abnormalities associated with diabetes declined; however, patients still are at risk for complications such as renal failure, myocardial ischemia, proliferative retinopathy (often leading to blindness), and neuropathic syndromes.

**HLA Associations with IDDM**

In population studies it was found that the serological specificities DR3 and DR4 were highly associated with disease and DR1 and DR8 were somewhat associated (47). DR3/DR4 heterozygotes had the highest risk, (48) and DR2 and DR5 were negatively associated (45,46). However this is not the complete story as in studies involving monozygotic twins, both having susceptible alleles, in only 30-50% of the cases did both

develop diabetes (49). From this it can be inferred that there must also be critical 'nongenetic' environmental triggers of disease.

As previously mentioned linkage disequilibrium between DR and DQ often makes HLA associations to disease cloudy. This was the case with IDDM, since with better technologies, such as RFLP, it was resolved that DQB1*0302 (β chain from DQ8) is actually the gene most highly associated with diabetes rather than DR4 or DR3 (50,51). However since DQB1*0302 is found in approximately 95% of individuals with the DR4 haplotype, the two genes tend to be linked together (52,53). DQB1*0302 pairs with DQA1*0301 to create the DQ8 molecule. Individuals who are homozygous for DQ8 have a relative risk of eight to twelve of developing IDDM. Another DQ haplotype associated with IDDM is DQ2 (relative risk of disease is three to five) (41). DQ2 is formed by the pairing of DQA1*0501 with DQB1*0201. The highest risk HLA group, the DR3/DR4 heterozygotes (relative risk of five to thirty five), was genetically mapped as individuals who are DQ2/DQ8 (DR3 DQ2/ DR4 DQ8) (41). The protective or negatively associated alleles were also due to DQ, in this case DQ6 (more specifically DQA1*0102 / DQB1*0602). The relative risk is less than one even if the DQ8 is present (41).

One of the factors responsible for the acceptance of the theory that DQB1*0302 confers increased susceptibility to IDDM was the discovery that lack of an aspartic acid (Asp) at position 57 of the DQ β chain may also be involved in susceptibility (54). Further to this was the discovery that 96% of diabetics are nonAsp on both DQ alleles as compared to only 19% of healthy controls (55). Biochemical and structural studies have proven that residue 57 is part of the fifth pocket in the peptide binding groove (56). DQβAsp57 also is important in the formation of a salt bridge with DQαArg79 . These two residues form hydrogen bonds with the antigenic peptide, thus helping to increase the affinity of the peptide for the class II (57,58). βAsp57 is located in a pocket of the peptide binding groove and its importance has been demonstrated as a substitution of βAsp57 with serinine leads to decreased peptide affinity and βAla57 to Asp prevented binding of certain

peptides (59,60). These structural features of DQ may be related to the autoimmune nature of diabetes, as if the peptide binding groove is sufficiently changed to allow for the presentation of self peptides that normally should not bind class II.

There is also evidence for substitutions of amino acids with the DQ α chain of susceptible alleles (61,62). In DQA1*0301 (DQ8), and DQA1*0501 (DQ2) an arginine (Arg) is found at position 52. It is thought that DQαArg52 is associated with disease simply through the disequilibrium with the corresponding DQB1 alleles.

Recently the hypotheses that trans-heterodimers between DQ2 and DQ8 account for the high susceptibility of DQ2/DQ8 heterozygotes has come into vogue (41,63). This means that α and β chains from alleles in trans to one another are pairing to form class II heterodimers. Possible trans-heterodimers between DQA1*0301/DQB1*0201 (α chain from DQ8 and β chain from DQ2) and DQA1*0501-DQB1*0302 (α chain from DQ2 and β chain from DQ8) have been implicated in Caucasians (64), Afro-Caribbeans (65), and Chinese (66,67) populations as a marker of increased risk. This is possibly due to a dosage effect, whereby an individual of this haplotype may potentially express DQ2 (DQA1*0501 / DQB1*0201) molecules, DQ8 (DQA1*0103 / DQB1*0302) molecules (associated in cis to one another ), as well as two different DQ2 / DQ8 heterodimers from the trans-pairing mentioned above (41).

As with most things in science the βnonAsp57 association with diabetes is not absolute, since most Japanese IDDM patients have the Asp molecule in position 57 (47,68). HLA molecules most often seen in Japanese patients are DQA1*0301 together with DQB1*0303 and DQA1*0301 with DQB1*0401, showing that the MHC correlation to IDDM susceptibility may vary in some cases between different races.

Even though the HLA correlations with IDDM are convincing, there are populations of diabetics that do not have these susceptibility alleles indicating that our understanding of IDDM susceptibility is incomplete. There is also evidence in the literature that the class I

region may be involved in determining the rate of β-cell destruction (e.g. age of onset of disease) (69,70)

As previously mentioned there are HLA alleles that confer protection against diabetes -- namely DQ6 (DQA1*0102 / DQB1*0602 in Caucasians and Blacks, DQA1*0103 / DQB1*0601 in Japanese). This protection seems to be dominant in that DQ2 / DQ6 or DQ8 / DQ6 individuals do not usually develop diabetes (53,71) Because the connection between diabetes and HLA susceptibility is so obscure, a clear explanation as to why some HLA alleles bestow protection has yet to be unveiled.

**Models of IDDM Pathogenesis**

Because the disease mechanisms of diabetes are not fully elucidated, the current models of disease pathogenesis are purely speculative thus far. Nepom proposed a determinant capture model in which protective alleles bind diabetogenic peptides with a higher affinity and thereby render them harmless (64). This was extended by Sheehy to include high affinity binding of diabetogenic peptides by protective alleles during thymic negative selection so that T cells recognizing this combination would be destroyed. Susceptible alleles would bind diabetogenic peptides with a lower affinity, allowing T cells with reactivity to these peptides to escape negative selection (61).

**Molecular Mimicry**

But what triggers the T cells to react against self antigens? Molecular mimicry is the phenomenon whereby a T cell is activated in response to an environmental antigen (e.g. a virus). The antigenic (viral) peptide the T cell recognizes has sufficient similarity to a self peptide so that even after the infection is cleared, the T cell continues to react; only now the focus is on the self antigen (72). There is evidence that this is occurring in IDDM. Islet-cell antibodies as well as full scale diabetes have been reported following congenital infection with rubella virus (73,74). The E1 protein of rubella virus contains a segment of

five amino acids (GPPAA) which is identical to a segment between positions 53-57 of the DQB1*0302 allele (DQ8β chain). This motif is also seen in Epstein-Barr virus (EBV) (6). The presence of anti-Coxsackie B4 (CB4) antibodies has been detected in newly diagnosed diabetics (75,76). Further to this, peripheral blood mononuclear cells that were responsive to glutamic acid decarboxylase (GAD) peptide (aa 247-279), also proliferated in response to a homologous peptide from the CB4 P2-C protein (aa 32-47) (77). The significance of these potentially cross-reactive epitopes and how they change normal immunological functions is still unclear; however, some evidence supports molecular mimicry as a model for IDDM susceptibility.

**Diabetic Autoantigens**

Since diabetes is the result of an autoimmune response that destroys pancreatic β cells, there must be specific antigens in these cells that induce and propagate this autoimmune response. Insulin or proinsulin and glutamic acid decarboxylase 65 (GAD) are examples of such antigens (78,79). Because β cell autoantibodies are present years before the onset of diabetes, they are used as a marker of IDDM development; however, there are first degree relatives of diabetics with circulating autoantibodies that never develop disease (80). GAD65 was originally identified by immunoprecipitation of labeled islets using sera from IDDM patients (81). There are two isoforms of GAD; GAD67 is highly homologous to GAD65 but is not a diabetic autoantigen (82). The GAD65 (from here forward referred to as GAD) isoform is expressed in human β cells, and antibodies against this protein are the earliest detectable sign of disease; autoantibodies to GAD are found in 80% of patients (80). T cell epitopes from GAD have also been identified (83) but this work is by no means complete.

Another autoantigen is the hormone insulin or its precursor proinsulin. Upon translation and transport into the rough endoplasmic reticulum (ER) of the β cell, preproinsulin is cleaved by a signal peptidase. This removes the 24 aa leader peptide from

the N terminus, which is known to target the hormone into the secretory pathway. Proinsulin results from this first cleavage, and following translocation from the ER to the Golgi complex it forms disulfide bonds and assumes its native structure. In the storage granules of the β cell proinsulin is further cleaved at two positions by specialized proteases to yield insulin and C-peptide. The C peptide is a 31 aa segment arising from the center of the proinsulin chain, and it is stored in the granules and excreted along with insulin. C peptide can be monitored in the serum of patients, and it provides a direct measure of the amount of endogenous insulin being synthesized (42). Approximately 50% of newly diagnosed diabetics have autoantibodies to insulin (84), but autoantibodies to proinsulin are also very prevalent. Insulin reactive T cells have been found in pre-diabetic individuals as well as in newly diagnosed IDDM patients (85).

Other human β cell autoantigens have also been described and the list continues to grow. A tyrosine phosphatase IA-2, formerly known as ICA 512, has been identified using the same technique as for GAD (86). The list also includes heat shock protein (hsp) 65 and a number of unidentified β cell antigens including a 38 kDa protein that have been shown to simulate T cells from IDDM patients(87).

**Non-obese Diabetic Mice**

Non-obese diabetic mice (NOD) are a well accepted animal model of human IDDM. These mice develop diabetes after 13 weeks of age, with the incidence in females being approximately 60% higher than in males (43). In general when NOD mice have been crossed onto any other inbred mouse strain, the F1 progeny do not develop diabetes. As with humans, disease susceptibility in NOD mice is a polygenic trait, but it most closely linked to the MHC -- only animals that are homozygous for NOD H-2 will develop diabetes (88,89). McDevitt has also identified a lack of Asp57 on the $IA^{NOD}$ beta chain that parallels the findings in humans (54,90). To date there has been more success in identifying T cell epitopes in autoantigens in the NOD mouse than in humans, as

experimental manipulation is easier in the animal model (91). Caution should be exercised because has not been proven that the disease mechanism in the NOD is the same as in human IDDM.

**Strategies for Immunotherapy Using Defined Peptides from Autoantigens**

The pursuit of T cell epitopes from islet β cell autoantigens is multifaceted. Two applications of this knowledge are the establishment of standardized peptide reagents for the detection of individuals in early phases of β cell loss, and the specific blocking or slowing of the autoimmune disease progression. With respect to the latter, there are several approaches which could be taken. The first, a new concept, called altered peptide ligands (APLs), could possibly be implemented in diabetic immune therapy or prevention trials. The central idea of altered peptide ligands is that when T cell epitopes are known they can potentially be modified slightly in order change the 'nature' of the immune response. Altered peptide ligands usually have a one amino acid difference near the area of the peptide that is recognized by the T cell (92). The result is that the altered peptide can function as a partial agonist (e.g. partially activate the T cell) or antagonist (block activation totally) (25). Partial activation has been demonstrated in a Th1 clone that produced IL-2 receptor but failed to secret IL-2 in response to an altered peptide ligand (93). In a separate experiment, after activation with an altered peptide, a Th2 clone produced cytokines but did not undergo proliferation (94). If pre-diabetics or those in the early stages of β cell destruction were identified through T cell responses to diabetes autoantigens or peptides, perhaps altered peptides based on these epitopes could be administered as a way of modifying or 'thwarting' the autoimmune response and thus preserving the islets. A second therapeutic approach is the use of DQ8 restricted T cell epitopes bound to soluble class II. Like the APL therapy, this therapy is based on the idea of specifically inactivating only the autoantigen-reactive T cells (clonal anergy). The theory is based on the idea that the peptide and soluble class II would be able to form the

trimolecular complex (TCR/peptide/MHC) with autoreactive T cells but because there is no APC present, T cell activation would not occur. This approach has been successful in preventing experimental allergic encephalomyelitis (EAE) in susceptible mice (95). The peptide could be exogenously loaded onto the class II molecule of DQ8 homozygous typing cells (e.g. BSM cells -- an EBV transformed B cell). Purification of class II molecules is a relatively simple procedure (96) involving immunoprecipitation of the class II. A similar tactic to the above mentioned would be the creation of a monoclonal antibody that recognizes the peptide from the autoantigen in the peptide binding groove of the DQ8 class II. On such monoclonal antibody exists (97). This monoclonal antibody sees an Eα peptide on the $IA^b$ molecule of mice. It was generated by immunizing class II incompatible mice with activated spleen cells. A possible strategy for creating such an antibody involves once again exogenously loading the identified peptide onto DQ8 class II homozygous typing cells and using this as a murine inoculum to generate the monoclonal antibody. This approach is labour intensive but would be easier to accomplish in "bulk" as would be required in a therapeutic application. There are some draw-backs to these types of immunotherapies. They all rely on the introduction of the therapeutic compound (e.g. monoclonal antibody or peptide on class II or APL) into the peripheral blood. This could be problematic in that reactive T cells tend to be concentrated in the islets and it would require huge doses of these compounds to be of remedial value in the pancreas. Another problem with using peptides from autoantigens is that recently it was discovered that certain peptides from myelin basic protein were in fact T cell epitopes but they were not immunogenic; while other peptides (Ac1-9) were highly immunogenic but remained bound to the class II only transiently (98). In fact the administration of the Ac1-9 peptide into encephalitogenic TCR transgenic mice had no impact on the T cell repertoire and did not instigate clonal deletion of autoreactive T cells (99,100). Given these concerns another possible mode administration has been tested. In a study whereby multiple sclerosis patients were fed myelin, the number of myelin basic protein reactive T cells were reduced

(101). Oral therapy has also been implicated in rheumatoid arthritis (using type III collagen) (102).

Although the idea of therapy directed solely towards autoreactive T cells is attractive, it is highly speculative. A first stage in the design of simple diagnostic reagents requires that the T cell epitopes be identified. In mice it is possible to stimulate bulk populations of T cells by in vivo immunization and then look a the call responses to an overlapping set of peptides. Unfortunately this cannot be done in humans. There are several approaches that can be taken to identify such epitopes in humans. The first is to create T cell clones from diabetic patients. This technology relies on circulating T cells that are autoreactive. These occur relatively infrequently, and the cloning method is very tedious and lengthy. Since IDDM is most highly associated with DQ8, it would be useful to identify T cell epitopes which are restricted to DQ8. However, in humans DR restricted responses seem to outnumber and will therefore likely mask DQ restricted responses, at least for circulating lymphocytes (103). Another more recent approach is to use human MHC class II transgenics to map DQ8 restricted epitopes. This means that the mice can be immunized with islet autoantigens, but at the same time the CD4 immune responses will be entirely restricted to human class II molecules (provided that the mouse class II has been first 'knocked out').

**Transgenic Mice**

There has been some success in the use of human MHC class II transgenic animals in the study of IDDM. Currently two methods of making transgenics are employed; injecting genomic DNA or cDNA. One successful DR4 transgenic used a chimeric molecule consisting of the human DR4 class II peptide binding groove (human $\alpha$1 and $\beta$1 domains) linked to the mouse $\beta$2 domains ($\beta$2 providing the recognition site for mouse CD4) plus mouse transmembrane and cytoplasmic signal sequences (104,105)(see also chapter III). These animals could generate DR4 restricted CD4 T cell response to proteins

and peptides and, represent one promising approach to the study of immune responses using human class II cDNA transgenic mice. Another pioneering group working on human class II transgenics was able to demonstrate T cell responses in animals that were transgenic for genomic human DQ6 (106). These two approaches are based on two different philosophies ; with the central question being whether or not mouse CD4 can successfully interact with human class II with sufficient affinity for T cell maturation and education to occur in the thymus. A key related issue is whether or not mouse IA and/or IE will 'out-compete' the human class II molecules, so that even if both mouse and human class II molecules are present in the mouse, nearly all of the CD4 T cells will end up being restricted to the mouse class II likely because of the higher affinity of mouse CD4 for mouse class II molecules. This in fact may have been the case with one set of experiments. David et al. have made human DQ8 genomic transgenic mice and were unable to elicit any T cell responses that were restricted to DQ8 until the endogenous (mouse ) class II molecules were removed (107) (see chapter IV).

**Experimental Design**

The main goal of this thesis was establish the tools needed in order to quickly map DQ8 restricted T cell epitopes for the diabetes autoantigens GAD and proinsulin. Two different human class II transgenic models, chimeric mouse IA / human DQ8 (utilizing cDNA) and wild-type DQ8 (utilizing genomic DNA), are described in chapters three and four respectively. The genomic DQ8 NOD mice in chapter four were intercrossed with NOD class II knockout mice in order to eliminate T cells restricted to $IA^{NOD}$. Chapter five discusses the creation of a eukaryotic expression vector (using EBV sequences) for the purpose of generating APC lines that are permanently transfected with GAD, human proinsulin, or other antigens. The goal of this work was to develop an alternate transfection based method for mapping T cell epitopes without requiring large sets of overlapping peptides. These three projects generated several collaborations. In chapter

six the results of one such collaborative project is described. It was designed to answer questions about the existence of trans-hetrodimers in the NOD class II knockout mice.

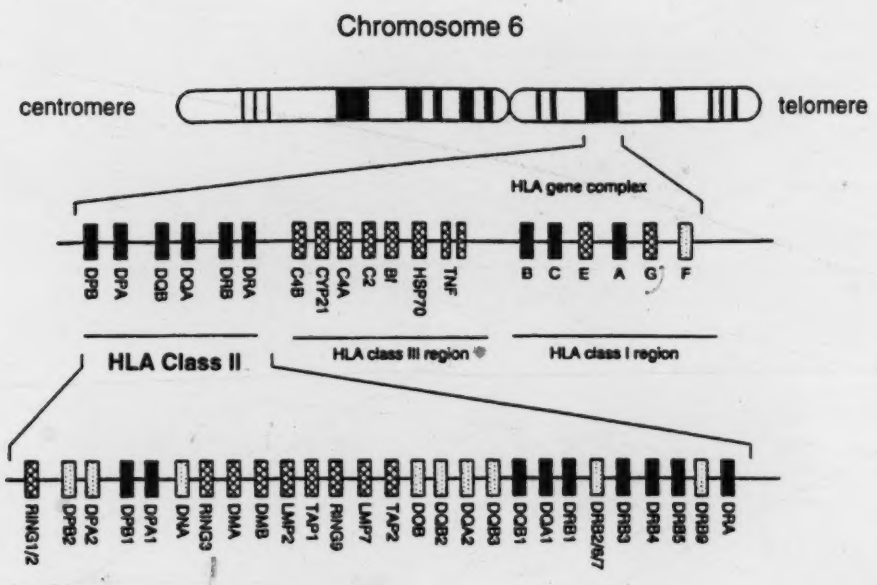



**Figure 1.1.** Detailed map of the human MHC region. The HLA gene complex is located on the short arm of chromosome 6. Class II genes are located on the centromeric end, class I on the telomeric end, and class III genes are in the middle. In the class II region, DP is separated from the DR and DQ genes by LMP, HLA-DM, and TAP genes. In the lower right-hand part of the figure, segments which encode class II $\alpha$ or $\beta$ chains known to be expressed in at least some alleles are shown as solid boxes. Adapted from: Throsby and Ronningen. 1993. Particular HLA-DQ molecules play a dominant role in detrmining susceptibility or resistance to Type 1 (insulin-dependent) diabetes mellitus. *Diabetologia*. 36:371-377.

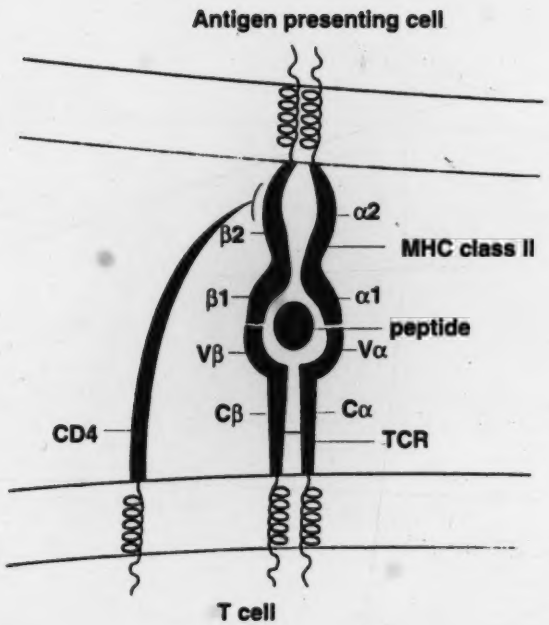



**Figure 1.2.** Schematic diagram of the ternary complex formed between a T cell, antigen presenting cell, and peptide. The peptide on the class II molecule is recognized by the TCR . Interaction between the TCR and class II is not fully defined but is thought to occur through the β1, α1 domains of the class II and the Vβ, Vα domains of the TCR. The CD4 molecule interacts with the β2 domain of the class II. Adapted from: Kuby. 1997. Immunology. 3rd Ed. W.H. Freeman and Company; page 99.

# CHAPTER II

## MATERIALS AND METHODS

### 1. Mice and reagents

$IA^{NOD}$ $DQ8^{+/+}$ mice were provided by Dr. Linda Wicker of Merck Research Laboratories, Rahway, NJ (unpublished). The $IA_{\beta}{}^{NOD}$ knockout mice were supplied by Dr. Tony Jevnikar of the University of Western Ontario, London, ON (manuscript in preparation). Purified OVA (A3686), SEA (S9399), SEB (S4881), and TSST-1 (T5662) were obtained commercially from Sigma. All restriction enzymes were purchased from New England Biolabs (NEB) and human GAD and human proinsulin were made using recombinant technology by Simon Rabinovich in John Elliott's lab. The FITC conjugated F4/80 , anti-mouse macrophage antibody (108) was purchased from Cedarlane and LY-2 , anti-mouse CD8 antibody was obtained from Pharmingen (01044A). The $OVA_{323-339}$ and $GAD_{273-285}$ peptides as well as their corresponding hybridomas 47.28 and 33.1 were generously provided by Dr. Wicker.

### 2. Cells

BSM (GM06821A) and WT 49 (GM03098A) cells were obtained from the Human Mutant Cell Repository. These are Epstein Barr Virus (EBV) transformed human B cells that were generated from the offspring of a consangious marriages, unique because they are homozygous for DR4, DQ8 and DR3, DQ2 respectively. These cells were maintained in complete RPMI-1640 [20% heat inactivated FCS, 2mM glutamine, 100U/ml penicillin, 100µg/ml streptomycin]. The two hybridoma cells, 33.1 and 47.28, were created by inoculating DR4 chimeric transgenic mice with the $GAD_{273-285}$ peptide (83) and genomic DQ8 transgenic NOD mice with $OVA_{323-339}$ peptide respectively. Both T cell hybridomas

were maintained in RPMI-1640 supplemented with 10% FCS, 50 μM 2-ME. B cell hybridomas 10-3.6 (ATCC TIB 92 ref 3) (109), Y-3P (ATCC HB 183) (110), RA3-3A1/6.1 (ATCC TIB 146) (111), H57-597 (ATCC HB 218) (112), GK1.5 (ATCC TIB 207) (113), as well as HeLa cells (ATCC CCL2) and COS-7 cells (ATCC CRL 1650) (114) were obtained from American Type Cell Collection (ATCC). The GAD-6 monoclonal antibody was obtained from the Developmental Studies Hybridoma Bank, University of Iowa (115). The monomorphic DQ8 monoclonal antibodies, FN81.1 and SPVL3 were generously supplied by Steinar Funderud, Norwegian Radium Hospital (116), Oslo Norway and Hergen Spits, Netherland Cancer Institute (117) respectively The B cell hybridomas were grown in bulk cultures, antibodies purified by affinity chromatography over protein A sepharose, and conjugated to FITC using standard methods (118).

### 3. Constructs and vectors

The EBOpLPP (119), SrαSD7 (120), EBO-pcD-Leu2 (121), and pHEF1αBSD7 (unpublished) vectors, as well as the cDNA encoding $IA_\beta^k$ and $IA_\alpha^k$ (cloned in the vector pSSD7) were kind gifts from Dr. D. Denney (Genitope Inc. CA). GAD cDNA was previously cloned in John Elliott's lab. Green fluorescent protein (GFP) was engineered as previously described (122,123). Ovalbumin (OVA) cDNA was obtained by PCR of the original pOV230 clone (124). Full length cDNAs encoding $IA_\alpha^d$ and $IA_\beta^{g7}$ were obtained by screening a lambda phage library made from NOD splenocytes (MMID 516 1995, L. Purdy and J. Elliott, unpublished). The minimal coding regions for each of the NOD chains were engineered to have 5' Xba I and 3' Not I sites using PCR (primers for the α chain were 5' -TCTAGACCACCATGGCCGTGCAGCAG and 3' -GCGGCCGCTCATAAAGGCCCTGGGTGT, for the β chain were 5'-TCTAGACCACCATGGCTCTGCAGATCCCC and 3' -

GCGGCCGCTCACTGCAGGAGCCCTGC). These coding regions were force cloned into the Xba I/ Not I sites of pSRαSD7.

**4. Purification of murine genomic DNA from tail preparations**

Each tail (0.5cm from the end of the tail of 14 day old mice) was harvested into 350µl of digestion buffer [100mM NaCl, 100mM EDTA, 50mM Tris HCl pH 8.0, 1% SDS] and incubated at 55°C overnight with 25µl Proteinase K (10mg/ml in 50% glycerol). The digestion was shaken periodically. When digestion of the tissue was complete, 125µl of saturated NaCl was added and the tubes incubated on ice for 15 minutes, pelleted, and the supernatants precipitated with an equal of isopropanol.

**5. PCR of genomic DNA**

The PCR reactions were completed on a PTC100 Programmable Thermal Controller (MJ Research Inc. Watertown Mass., U.S.A) using standard techniques for *Taq* polymerase (125). *Taq* polymerase was prepared on our laboratory using standard techniques (126). The following was combined in a Perkin Elmer PCR tube (N8010540): 36.8µl $H_2O$, 5µl 10x *Taq* buffer [50mM KCl, 10mM Tris HCl pH 8.4, 200µg/ml gelatin, and 2.5mM $MgCl_2$], 2µl dNTPs @ 2.5mM each, 2µl of tail DNA prepared above, 2µl each the of 5' and 3' primer (12.5µM), and 0.2µl of *Taq* polymerase. Primers were as follows: DQ8β GCCTTAGAGATGTCTTGGAAGAAGGCTTTG and GGAGATGGCGACATTGGGCTCCACTCGCCGTGCA; DQ8α GAGACCAGGATGATCCTAAACAAAGCTCT and GAACACAGTCGCTTGAGGAACCTCATTGGTAGC. Each cycle (30 in total) consisted of 94°C for 15 sec, 60°C for 30 sec, 72°C for 2.5 min.

**6. Purification of lymphocytes from spleen, lymph node and peripheral blood**

In sterile RPMI-1640 the spleen or lymph node was ground using the frosted edge

of two glass slides (heparinized blood was diluted in PBS) and gently overlaid onto 3 ml of room temperature Lympholyte-M (Cedarlane-CL5030). The suspension was centrifuged at 2000 rpm for 20 minutes at room temperature. One milliliter above and below the lymphocyte interface was collected into a clean tube and the volume diluted up to 10 ml using RPMI. The cells were pelleted at 1000 rpm for 10 minutes and then resuspended in 2 ml of proliferation media. Splenocytes were irradiated at 3000 rads in a Gammacell 40 Irradiator (Nordion International Inc. Kanata, ON).

**7. Cell Staining and FACS analysis**

Cells were washed once with Hanks Buffered Salt Solution supplemented with 10mM Hepes, 5mM EDTA, 5% FCS (HBSS). The cell pellet was resuspended in HBSS containing the appropriate FITC conjugated antibody (1:1000 dilution; stock ≈ 1 mg/ml), and incubated for 20 minutes on ice. HBSS was added to bring the volume up to 1 ml, and this suspension was centrifuged through an underlay of 1 ml FCS. The stained cells were resuspended in 200µl HBSS and analyzed by FACScan (Becton Dickinson).

**8. Dot blot analysis**

Murine genomic DNA (10µg prepared from tail digestion) was mixed with NaOH (final concentration 0.3M) in a 200µl volume. The DNA was incubated at 60°C for one hour and then neutralized with an equal volume (200 µl) of 2M $NH_4OAc$. The treated DNA was applied a pre-wet nitrocellulose (Schleicher & Schuell BA-85) membrane using a Minifold I Microsample filtration manifold (Schleicher & Schuell, Keene, N.H.) and the DNA cross-linked using a UV Stratalinker 1800 (Stratagene La Jolla, CA). The blot was pre-hybridized [50% formamide, 200 µg/ml salmon sperm DNA, 5x SSPE, 0.1% SDS, 5x Denhardt's, 200 µg/ml yeast tRNA] for 1 hour at 42°C. The blot was hybridized [50% formamide, 200g/ml salmon sperm DNA, 5x SSPE, 0.1% SDS, 5x Denhardt's, 200 µg/ml yeast tRNA, 10% dextran sulfate] overnight at 42°C with pre-boiled $^{32}P$ labeled

DQ8 probe that was made as previously described (127). The following day the blot was washed extensively [0.2x SSPE, 0.1% SDS in $H_2O$], dried and exposed for 2 hours. The probe was made from the DQA1*0301 cDNA insert excised from the clone SrαSD5 51QB2 (Dan Denney unpublished) using EcoRI and Not1.

**9. Lymph node cultures of antigen primed T cells**

Mice were inoculated in the hind foot pad using 50µl OVA, GAD or proinsulin (37.5µg total protein) emulsified in an equal volume of Freund's Adjuvant Complete H37 Ra (Difco-3113-60). Ten days later the popliteal lymph nodes were removed and purified over Lympholyte-M as previously described. Cells were resuspended in proliferation media [RPMI-1640 with 1% Nutridoma (Boehringer Mannheim), 50µM 2-ME, 50ug/ml gentamicin and 2mM glutamine] at a concentration of $3x10^6$ cells/ml and 100 µl was added to a 96 well plate (Falcon U bottom - 3077) in triplicate. Cells were incubated in the absence (background stimulation control wells) or presence (experimental wells) of varying concentrations of the whole protein. Blocking of T cell stimulation was completed using the monoclonal antibody SPVL3 (10µg/ml).

**10. Thymidine uptake**

Duplicate cultures of the above were established, incubated for 72 hours, and 1µCi methyl $^3H$ thymidine (Amersham TRK 120) in 20µl proliferation media added to each well. After 18 hours the wells were harvested onto a nylon filter using a Micromate 196 96 well cell harvester (Canberra Packard Canada). Each well was cut out of the filter and immersed in Ecolite scintillation fluid (ICN - 882475) and the CPM read on a Beckman L56000TA counter.

**11. Superantigen stimulation**

Splenocytes from a $IA_\beta^{-/-}$ $DQ8^{+/+}$ and a $IA_\beta^{-/-}$ mouse were isolated as described

above. Cells ($3x10^5$) were incubated in a 96 well U bottom plate in a final volume of 130µl alone (background negative control), with varying concentrations of SEA, SEB, or TSST-1, or with 1µg/ml Con A (Sigma C0412) for the positive control. The amount of IL-2 accumulated in 75µl of the supernatant was measured after 48 hours by the ELISA method described below.

## 12. ELISA for IL-2

Ninety six well plates (Costar EIA/RIA Flat bottom, high binding - 3590) were coated overnight at 4°C with 100µl of 2µg/ml rat and mouse IL-2 (PharMingen-18161D). The plates were washed 4 times with wash buffer [$H_2O$ 0.05% Tween 20] and blocked for one hour at room temperature using 3% bovine serum albumin (BSA) in PBS. Stimulation supernatant (75µl) was added and allowed to incubate for one hour at room temperature. The plates were washed four times with wash buffer and 125µl of 1µg/ml secondary antibody (PharMingen biotin anti-mouse IL-2 - 18172D) added and incubated wrapped in aluminum foil for one hour at room temperature on the shaking platform. Again the plates were washed four times with wash buffer and 100µl of a 1:2000 dilution of Streptavidin AKP (PharMingen - 13043E) added to each well. The plates were incubated in the same conditions as with the secondary antibody. The plates were washed again and 100µl of Alkaline Phosphatase substrate (Pierce - 37620) added and incubated in the dark until sufficient colour developed. The reaction was stopped using 50µl of a 2M NaOH solution and the plates read at 405 nm on a Molecular Devices UV Max (U.S.A.) plate reader. The IL-2 concentration in U/ml ($3.75x10^7$U/mg) was determined using a standard curve.

## 13. Electroporation of BSM cells

BSM cells were grown to a cell density of $1x10^6$ cells/ml and washed once with HBSE [20mM Hepes pH 7.0, 0.75mM $NaHPO_4/NaH_2PO_4$ pH 7.0, 138mM NaCl, 5mM

KCl, 5.5mM Dextrose]. Each electroporation used 20μg of vector DNA (purified on a CsCl/Ethidium gradient as described below) and $1x10^7$ cells in 400μl HBSE. The cells were electroporated at 210volts and 960μFd. Following electroporation the cells were cultured in complete media at 37°C and 5% $CO_2$ and slowly selected using hygromycin B (Sigma H9773). Starting at 50μg/ml the hygromycin was increased by 50μg/ml every 48 hours until the concentration was 200μg/ml. The cells were maintained at this concentration and kept at a density approximately $5x10^5$ cells/ml.

**14. Stimulation of OVA and GAD T cell hybridomas**

T hybridoma cells were grown to a density not exceeding $1x10^6$cells/ml in RPMI-1640 supplemented with 10% FCS, 50μM 2-ME, 2mM glutamine, 100U/ml penicillin and 100μg/ml streptomycin, counted and centrifuged at 1000 rpm for 10 minutes at room temperature. The T cells were then resuspended in proliferation media at a concentration of $1x10^6$ cells/ml. APCs (BSM cells or splenocytes irradiated at 8000 or 3000 rads respectively) were resuspended in proliferation media (concentration $2x10^6$ cells/ml). One hundred microliters of each lymphocyte preparation was added to a 96 well plate (Falcon U bottom - 3077) with varying concentrations of peptide or whole antigen to give a final volume of 230μl. Background wells were T hybridoma cells and BSM cells but no antigen. The positive controls were H57-597 bound to the 96 well plate or PMA (Sigma P8139) and Ionomycin (Sigma I0634). H57-597, an anti-mouse TCR monoclonal antibody, was bound at a concentration of 10μg/well for one hour at 37°C and the T cells added and incubated as the experimental wells. PMA and Ionomycin were added the T hybridoma cells at a final concentration of 10ng/ml and 1μM respectively.

Stimulations using the BSM transfectants were completed in the same manner as described for the testing of the hybridomas. When higher ratio stimulations were completed the total cell number per well was kept constant at $3x10^5$.

**15. Stimulation of OVA primed transgenic T cells using BSM transfectants**

$IA_{\beta}^{-/-}$ $DQ8^{+/+}$ Mice were inoculated in the hind foot pad using 50µl OVA (25µg total protein) emulsified in an equal volume of Freund's Adjuvant Complete H37 Ra (Difco-3113-60). Ten days later the popliteal lymph nodes were removed and purified as described above. Cells were counted and the volume adjusted so that the concentration was $1x10^6$cells/ml. Simultaneously the transfected BSM cells were counted and resuspended in proliferation media to a final concentration of $2x10^6$cells/ml and irradiated at 8000 rads. One hundred 100µl of each lymphocyte population was added to a 96 well plate (Falcon U bottom - 3077) in triplicate and incubated in the absence (test wells) or presence (positive control wells) of 6µM $OVA_{323-339}$ peptide.

**16. Western blots of BSM transfectants**

BSM transfected cells ($1x10^6$) were washed once in wash buffer [150mM NaCl, 10mM Tris HCl pH 7.5, 0.2% NP40] and then incubated on ice for 5 minutes in lysis buffer [150mM NaCl, 20mM Tris HCl pH 7.5, 1% NP40]. Cellular debris was pelleted and the supernatant mixed with 6x SDS/sample buffer (128). Samples were boiled for 5 minutes before loading onto a 10% polyacrylamide gel and electrophoresed on a Biorad Mini-Protean II Dual Slab Cell (128). Biorad broad range SDS-PAGE standards (161-0317) were dissolved in sample buffer, boiled and loaded as sample wells. Electrophoresis was carried out at 24mAmps for 45 minutes. Electroblotting (129) was performed using a Biorad Mini Trans-Blot Electrophoretic Transfer Cell and nitrocellulose membrane (Schleicher & Schuell BA85) in transfer buffer [25mM Tris HCl, 192mM glycine pH 8.0, 20% methanol] at 90 volts for one hour. Blots were blocked overnight in PBST 0.5% with 3% BSA (GAD blots) or 3% skim milk (OVA blots). Primary antibody incubation was as follows: OVA blots were incubated with 1:10 000 PBST 0.5% dilution of a polyclonal anti-OVA anti-serum, GAD blots were incubated at a 1:20 dilution with GAD-6 monoclonal antibody cellular supernatant for 20 minutes at room temperature with

shaking. Blots were washed twice for 10 minutes each in PBST 0.5%. Secondary antibody was a goat anti-mouse HRP conjugated Fc antibody (1:10 000 dilution) supplied in the Dupont detection kit (Renaissance Western Blot Chemiluminescence Reagent, Dupont NEL100). Incubation was for 15 minutes at room temperature with shaking. Blots were washed as follows: 10 minutes in PBST 0.3%, 10 minutes in PBST 0.1%, and 10 minutes in PBS. The blot was then developed using manufacturers direction with the above mentioned kit.

**17. Isolation of total cellular RNA**

The spleen from a C57BL/6 mouse was removed and flash frozen in liquid nitrogen. The tissue was homogenized in 6ml Tri Reagent (Molecular Research Center Inc.) and 2ml chloroform added. The tube was incubated on ice for 10 minutes with periodic shaking. The phases were allowed to settle out, centrifuged at 5000 rpm for 20 minutes at 4°C, and the aqueous phase was removed to a clean 2059 tube and precipitated using an equal volume isopropanol. The RNA was pelleted at 8000 rpm for 60 minutes at 4°C. The pellet was redissolved in 500 µl DEPC $H_2O$ (0.1% in distilled, deionized $H_2O$, autoclaved before use Sigma D5758), reprecipitated using 0.3M NaOAc and 2.5 volumes of 95% ethanol and finally resuspended in 100 µl DEPC $H_2O$ and snap frozen in 20 µl aliquots. The optical density at 260nm was read in order to determine the concentration.

**18. cDNA synthesis**

a. <u>First strand synthesis</u>

The following were combined in an eppendorf tube: 44µl DEPC $H_2O$, 8µl total cellular RNA (~10µg), 5µl oligo dT (1mg/ml). The tube was heated at 65°C for 2 minutes to denature the RNA and cooled on ice. Then 20µl dNTP's @ 10mM each, 10µl 10X RTase salts [1.0M Tris HCl pH 8.3, 0.5 M KCl, 0.1M $MgCl_2$, 0.1M DTT], 2µl Rnasin (40U/µl Promega), 6µl AMVRT (Molecular Genetic Research, Tampa, Fl.) was added

and the tube incubated at 41°C for 3 hours. The enzymes were inactivated by heating the tube at 65°C for 10 minutes, and it was cooled on ice. The reaction was diluted up to 1ml with 5mM Tris HCl pH 7.5 and centrifuged through a Centricon 100 (Amicon-4212) at 3000 rpm for 20 minutes at 4°C (completed twice). DEPC $H_2O$ was added to the centricon purified first strand DNA (volume ~ 50 µl) in order to bring the volume up to 176µl.

b. Second strand synthesis

The following were added to the first strand reaction; 20µl 10x SSR buffer [1.0M KCl, 200mM Tris HCl pH7.4, 100mM DTT, 50mM MgCl2], 2µl 15mM NAD, 2µl 10mM dNTP's @ 10mM each, 1µl RNase H ( 15U/µl Promega), 1µl E.coli DNA ligase (4U/µl NEB), 3µl DNA polymerase (10U/µl NEB). The tube was incubated overnight at room temperature. The reaction was transferred to a siliconized tube and 300µl TE [10mM Tris pH 8.0, 1mM EDTA] was added as well as an amount of spermine to equal a final concentration of 5mM. The cDNA was incubated on ice for 30 minutes, centrifuged for 20 minutes at 4°C, and washed with 1ml of spermine wash buffer [70% ethanol, 0.3M NaOAc, 10mM $Mg(OAc)_2$]. A second 30 minute incubation on ice was done, followed by a second centrifugation for 20 minutes at 4°C. The cDNA pellet was resuspended in 10µl DEPC TE.

## 19. RT-PCR of $IA_\alpha^b$ & $IA_\beta^b$

The following reagents were combined in a PCR tube (Perkin Elmer N8010540): 85 µl H2O, 10µl 10x cloned PFU buffer, 2µl dNTP's @ 10mM each, 1µl ds cDNA from above, 1µl 5' primer (100µM), 1µl 3' primer (100µM), 1µl cloned PFU (2.5U/µl Stratagene, La Jolla USA). Primers were α chain TCTAGACCACCATGCCGTGCAGCAGAGCT and GCGGCCGCTCATCCCGGCCCTGGGTGT; β chain TCTAGACCACCATGGCTCTGCAGATCCCC and

GCGGCCGCTCACTGCAGGAGCCCTGC. Each PCR cycle consisted of 94 °C for 25 seconds, 58°C for 30 seconds, 72 °C for 30 seconds. Thirty cycles were performed on a PTC100 Programmable Thermal Controller, MJ Research Inc. Watertown Mass., U.S.A.

**20. Cloning of PCR fragments**

The RT-PCR products were purified by adding 2ml 5mM Tris HCl pH 7.5 and centrifuging through a Centricon 100 (Amicon- 4212) at 3000 rpm for 40 minutes at 4°C. This was repeated and the products recovered. The entire amount recovered (~50µl) was phosphorylated by adding the following: 6µl 0.5M Tris HCl pH 7.4 0.1M $MgCl_2$, 3µl DTT (200µM), 0.6µl rATP (100µM), 1.2µl T4 polynucleotide kinase (10U/µl NEB) in two aliquots. This was incubated at 37°C for one hour and then cooled on ice. Next the ends of the PCR products were blunted and then ligated together using 0.3µl dNTP's @ 10mM each, 0.6µl rATP (100µM), 1.0µl T4 DNA ligase (400U/µl NEB), 1.0µl Klenow - DNA Polymerase large fragment (5U/µl NEB). The reaction was incubated overnight at 15°C. To stop the reaction 140µl TE and 10µl 10% SDS were added and the mixture phenol/chloroform extracted two times. Lastly the aqueous phase containing the purified PCR products was precipitated with 3M NaOAc and 95% ethanol, pelleted at 4°C, and resuspended in 31.5µl $H_2O$. The PCR products were digested with XbaI / NotI (sites incorporated into the PCR primers) and cloned into XbaI /NotI digested pSRαSD7 as above.

**21. DEAE transfection of COS -7 cells**

The following were to complete DME [DME 10% FCS 100 IU/ml penicillin, and 100 µg/ml streptomycin]: 10µg of each IA chain (CsCl/Ethidium bromide gradient purified), DEAE dextran 200µg/ml (Sigma D9885), chloroquine 100µM (Sigma C6628) in 3ml complete DME . The mixture was added to 30% confluent COS cells in 6 well plates (Falcon 3046) and incubated for four hours at 37°C and 5% $CO_2$. The cells were

shocked with 10% DMSO (J.T. Baker 9224-01) in DME and then given fresh complete media. At 24 hours the cells were removed from the plate with Trypsin EDTA (Gibco) and placed in fresh six well plates. Cells were analyzed after 72 hours. Following staining of transfected COS cells with FITC conjugated antibodies the cells were forced through a nylon mesh (45μm) in order to separate the COS cells as they have a tendency to clump.

**22. Electroporation of HeLa cells**

Cells were grown until confluent in complete DME at 37°C and 5% $CO_2$. For electroporation, cells were removed using Trypsin EDTA and washed once with HBSE. Cells ($1x10^6$) in 400μl HBSE were electroporated in a 0.4 cm cuvette with a Bio-Rad Electroporator set at 1.2kV and 25μF using 20μg of each plasmid (DNA purified over CsCl/ethidium bromide gradients). Cell were analyzed after 72 hours.

**23. General cloning methods**

a. <u>β agarase</u>

The excised bands containing the DNA insert or vector backbone were heated at 70°C in order to melt the agarose. The two were combined and the volume estimated and 10x βagarase I buffer added (NEB). The mixture was transferred to a 40°C water bath, 1U βagarase (concentration 1U/μl) per 200μl added, and incubated for a minimum of two hours. Following digestion the DNA was precipitated by: incubating on ice for 15 minutes, centrifuging the sample in order to pellet the carbohydrate, transferring the supernatant to a clean tube and precipitating with an equal volume of isopropanol isopropanol and 0.3M NaOAc.

b. <u>Ligation of DNA inserts</u>

Following precipitation of βagarse digested inserts and vector backbones, the DNA pellet was resuspended in 18μl of $H_2O$ and 2μl of 10x T4 DNA ligase buffer (NEB) was

added. T4 DNA ligase (0.2µl of a 400U/µl) was added and the mixture incubated at 14°C for a minimum of four hours.

c. Transformation of ligation

DH5α competent cells were thawed on ice and to 50µl aliquots, 5µl of each ligation was added. The tube was gently mixed and incubated on ice for 10 minutes. The bacteria were heat shocked at 37°C for 90 seconds and outgrown in 1 ml 2XYT media on the roller wheel for 30 minutes. The bacteria were pelleted and plated out onto LB 100 µg/ml ampicillin [1% bacto-tryptone, 0.5% bacto-yeast extract 1% NaCl 1.5% agarose] plates and incubated overnight at 37°C. Into 4 ml of 2XYT [1.6% bact-tryptone, 1% bact-yeast extract, 0.5% NaCl] a single bacterial colony was picked and incubated a minimum of 10 hours at 37°C on the roller wheel.

d. Alkaline lysis plasmid mini preparation

One milliliter of each overnight bacterial culture was added to an eppendorf tube. The culture was centrifuged for one minute at room temperature in order to pellet the bacteria and the supernatant was decanted. One hundred microliters of Solution I [50mM glucose, 10mM EDTA, 25mM Tris HCl pH 8.0] was added and the tube vortexed. The same amount of fresh Solution II [2.0M NaOH, 1% SDS,] was added and the tube vortexed again. The mini preps were then incubated at room temperature for 5-10 minutes to ensure complete lysis of the bacteria and shearing of the chromosomal DNA. One hundred microliters of Solution II [60ml 5M KOAc, 11.5ml glacial acetic acid, 28.5ml $H_2O$] was added. The tubes were vortexed to mix and incubated on ice for 15 minutes in order to neutralize the lysis reaction. The cellular debris was pelleted by centrifuging for 15 minutes at room temperature. The supernatant was transferred to a clean eppendorf tube and 30µl 3M NaOAc was added, followed by one volume of isopropanol. The DNA was pelleted at 4°C for 15 minutes and washed once with 70% ethanol. The pellet was allowed to air dry and then was resuspended in 100µl TE [10mM Tris HCl pH 8.0, 1mM EDTA pH 8.0]. The clones were mapped using restriction enzymes.

e. Large plasmid preparations

Clones determined to be positive after mapping the mini preps were grown up overnight in 200ml superbroth [1.2% bacto-tryptone, 2.4% bacto-yeast extract, 0.63% glycerol, 1.25% $K_2HPO_4$, 0.38% $KH_2PO_4$] with 100µg/ml ampicillin at 37°C with shaking. The bacteria was pelleted at 6000 rpm for 10 minutes. The supernatant was decanted and the pellet allowed to drain. Five milliliters of lysis buffer [50mM glucose, 25mM Tris pH 8.0, 10mM EDTA with fresh lysozyme] was added and the tube vortexed rigorously in order to resuspend the pellet. The mixture was incubated on ice for 10-20 minutes, followed by the addition of 10ml freshly made 0.2N NaOH, 1% SDS. After mixing the tube was incubated at room temperature for 20 minutes. To the preparation 7.5 ml of cold 7.5M $NH_4OAc$ was added; and again mixed well and incubated on ice for 10-30 minutes. The cellular debris was pelleted at 14 000 rpm for 30 minutes at room temperature. The supernatant was poured through a cheese cloth in order to remove floating debris and incubated with 25µl of a 10mg/ml RNase A (Boehringer Mannheim) solution at 37°C for one hour. One volume of phenol/chloroform was added and the tube shaken vigorously and subsequently centrifuged at 5000 rpm for 10 minutes to separate the phases. The aqueous phase was removed to a clean tube and precipitated with isopropanol. The precipitation was placed at -20°C for a minimum of 30 minutes and then pelleted at 15000 rpm for 10 minutes. After allowing the DNA pellet to dry slightly, it was resuspended in 1.5ml TE and transferred to an ultracentrifuge tube. Three milliliters CsCl (ICN biomedical Inc. Aurora, OH) stock solution [1.2mg/ml reference index 1.4155] and 100µl of a 10mg/ml ethidium bromide stock was added and mixed with a pasteur pipette. The tube was topped up with a $CsCl:H_2O$ (2:1), balanced, and heat sealed. The gradient was centrifuged at 55 000rpm overnight. The next morning the plasmid bands were quickly and gently extracted from the gradient using an 18 gauge needle and 3ml syringe. The ethidium bromide was extracted using NaCl saturated isopropanol. The volume is increased to 6ml using TE and the DNA precipitated with isopropanol. Following an

incubation at -20°C the DNA is pelleted and washed with 70% ethanol. The DNA was resuspended in TE and the optical density at 260nm read in order to calculate the concentration.

# CHAPTER III

## EARLY ATTEMPTS TO ESTABLISH TRANSGENIC MICE EXPRESSING MOUSE IA / HUMAN DQ8 CHIMERIC CLASS II MHC MOLECULES

### INTRODUCTION

In the quest to elucidate the etiology of various autoimmune disease scientists have begun to use human transgenic animals as model systems for studying human immunology. The role of HLA in autoimmune disease is well established (130,131) and researchers have begun using transgenic animals expressing disease associated HLA gene products in order to further our understanding of disease mechanisms. In the case of autoimmune diabetes, this includes DQ8 (DQA1*0301 and DQB1*0302), thus far the most important genetic factor in determining predisposition to disease (132). Currently there are two popular strategies for creating transgenic mice, by using cDNA or genomic sequences (104,106). In this approach we chose to use cDNA to encode a chimeric mouse IA / human DQ8 transgene.

Another variable to consider in this project was the interaction between the human class II and the endogenous mouse CD4 molecule. Opinions currently differ as to whether this cross-species binding can occur. Sasazuki reported DR51 and DQ6 transgenic mice generated CD4 T cells that responded to peptides on the appropriate human class II (106), and when the DQ6 mice were crossed onto a human CD4 transgenic mouse with the mouse CD4 'knocked-out', the DQ6 restricted immune response did not appear to be increased (133). A possible reason for this is that the human CD4 transgene was controlled by human regulatory elements that are not appropriately active in mouse CD4 cells.. Straub et al. reported both positive and negative selection of certain Vβ T cell subsets in HLA-DR3 transgenic mice; however, the efficiencies were relatively low,

suggesting that although the wild-type human class II can interact with mouse CD4, this association is not as effective as when both molecules are from the same species(134). Another school of thought argues that the interaction between the human class II β2 domain and mouse CD4 is insufficient to create an effective immune response (135-137). A reasonable solution to this barrier would be to use a chimeric human/mouse class II molecule (104,105) (figure 3.1). Zaller et al. have employed such a system in creating a transgenic mouse that has the peptide binding groove and T cell receptor interacting domains of human DR4 but has the mouse membrane proximal, CD4 binding, and cytoplasmic signaling domains (104). We elected to mimic such a system in the creation of DQ8 transgenic mice. This necessitated the use of cDNA rather than the genomic DNA, but this was favorable as cDNA required a few thousand bp and involved relatively straight forward cloning whereas the use of genomic DNA would have required many thousand bp and technically difficult cloning procedures. Whenever cDNA is used to construct transgenics a key issue becomes which promoter should be used to give tissue appropriate expression. A relevant cDNA expression vector called pDOI-5 has been constructed by Benoist and Mathis (138). The vector contains approximately 2200bp of upstream DNA from the IEα gene, together with a rabbit β–globin intron and rabbit β-globin polyadenylation signal. pDOI-5 was reported to give tissue and cell type expression like endogenous class II in 50% of transgenic animals (138); however, a significant difficulty is that before microinjection into mouse embryos the bacterial sequences of the plasmid must be removed from the rest of the DNA and with the restriction sites present in pDOI-5 this task is very difficult or impossible. With the general structure of pDOI-5 in mind a new vector called pAbSIP7 was constructed and human DQ8 / mouse IA chimeric cDNA elements were assembled, and these were used to generate transgenic mice.

RESULTS AND DISCUSSION

The cDNAs encoding chimeric DQA1*0301/$IA_{\alpha}^{nod}$ and DQB1*0302/$IA_{\beta}^{nod}$ (Dan Denney

unpublished) as well as the cDNA expression vector pAbSIP7 (figure 3.2) were designed and constructed by Dr. Dan Denney (figure 3.2). The 5' promoter / enhancer element (primers GGCTTAGGAGATCTGTGTCTGT and ACAGACACAGATCTCCTAAGCC) and 3' polyadenylation signal (primers ACGCGTGCGGCCGCGCGCATGCCAGTCCACCTGCCA and ATCGATCGCGACGTCCATCATCAGGAAGCAGAGA) in pAbSIP7 arose from PCR of genomic DNA isolated from the liver of a C57BL/6 mouse (Dan Denney unpublished). The upstream promoter sequence consists of a 2380 bp genomic segment located immediately upstream of the first exon of $IA\beta^b$. By utilizing a segment extending this far upstream of the exon, it was anticipated that the sequence would contain all of the necessary class II regulatory elements (e.g. the promoter and enhancer). The downstream region consisted of a 1548 bp genomic segment which occurs immediately downstream of the last exon of $IA\beta^b$, and contains the polyadenylation sequences for $IA\beta^b$ as well as potential downstream regulatory elements. In terms of a spicing element (e.g. splice donor/intron/splice acceptor thought to be necessary for proper RNA expression and transport), initially the genomic element of $IA\beta^{g7}$ lying between the first and second exons (but excluding any ATG codons) was placed immediately downstream of the $IA\beta^b$ promoter. However this failed to function (Dan Denney unpublished), and therefore it was replaced with the SV40 slice donor and acceptor elements from the SRαSD7 expression vector (120), as shown in figure 3.2. The remaining segments of the vector include the ampicillin resistance gene (bla), the polylinker (containing unique restriction enzyme sites to allow cDNA to be cloned into the vector), and a pUC-derived bacterial origin of replication. Following the cloning of DQ8 chimeric α and β cDNAs into pAbSIP7, the two plasmid DNAs were purified and the bacterial sequences were removed by digesting each plasmid with the restriction enzyme AatII followed by electrophoresis on a 0.5% agarose gel. The appropriate α and β bands were excised from the gel and the DNA recovered using an Elu-Quick DNA purification kit (Schleicher & Schuell). The

ethanol precipitated DNAs were resuspended in TE at 2-5μg/ml and pooled prior to microinjection into FVB/N mouse embryos by Dr. Peter Dickie (139).

Transgenic founder animals were identified by PCR analysis of genomic DNA purified from the tails of two week old mice using the following primers: α chain GAGACCAGGATGATCCCTAAACAAGCTCT and GAACACAGTCGCTTGAGGAACCTCATTGGTAGC; β chain GCCTTAGAGATGTCTTGGAAGAAGGCTTTG and GGAGATGGCGACATTGGGCTCCACTCGCCGCTGCA. Two separate PCR reactions were done, one for each chimeric cDNA. The next generation of offspring required only one PCR reaction as only animals that were positive for both the α and β chains were bred. Founders (primary positive transgenic mice) were bred to normal FVB/N mice. Positive offspring from this mating were bred together (e.g. brother sister matings). This is standard practice in the generation of transgenic lines. Two lines (from different founders) are never crossed as this could lead to higher copy number and/or instability of the transgene, since with each founder embryo the transgene integrates into a different area of the genome. An example PCR analysis of transgenic mice is shown in figure 3.3. These were F2 animals (offspring of the brother sister matings arising from the original founder animal #7) ; mouse #7-2 and mouse #7-9 were positive and mouse #7-8 was negative. In the F2 generation positive animals were then tested to see if they were homozygous or heterozygous for DQ8. Purified tail DNA (10μg) was dotted onto nitrocellulose membrane as described in the Materials and Methods. Homozygous animals were twice as bright as heterozygous when probed with a $^{32}P$ labeled probe that was created from the DQ8 α chain cDNA (figure 3.4).

The final test was to see if the transgenes present were functional and gave rise to expressed protein on the surface of cells which normally express class II. Splenocytes were isolated and stained with FN81.1 (116) or SPVL3 (117) [both monomorphic antibodies against human DQ molecules] and compared to staining patterns of

nontransgenic littermates. The positive control was BSM, human EBV transformed typing cell line that is homozygous for DQ8 (figure 3.5). The FACS profiles showed that both monoclonal antibodies were functioning as the BSM cells stain positive. Unfortunately, there was no difference in the staining of nontransgenic and transgenic littermates, indicating that the DQ8 chimeric cDNA although present was not being expressed on the surface of splenocytes. Similar F2 mice derived from all other founder animals (five lines in total) stained exactly the same, indicating that non-expression was not due to the integration of the transgene in this line but was more likely due to some failure of the expression vector itself.

At this point we were presented with two options; to go back and rebuild the vector to try to change the regulatory elements so that the cDNA would be expressed, or to begin a new collaboration that would allow us to study T cell epitopes to diabetes autoantigens with a different approach. The chimeric project was abandoned and we began working with genomic transgenic mice expressing wild type human DQ8 as described in the following chapter.

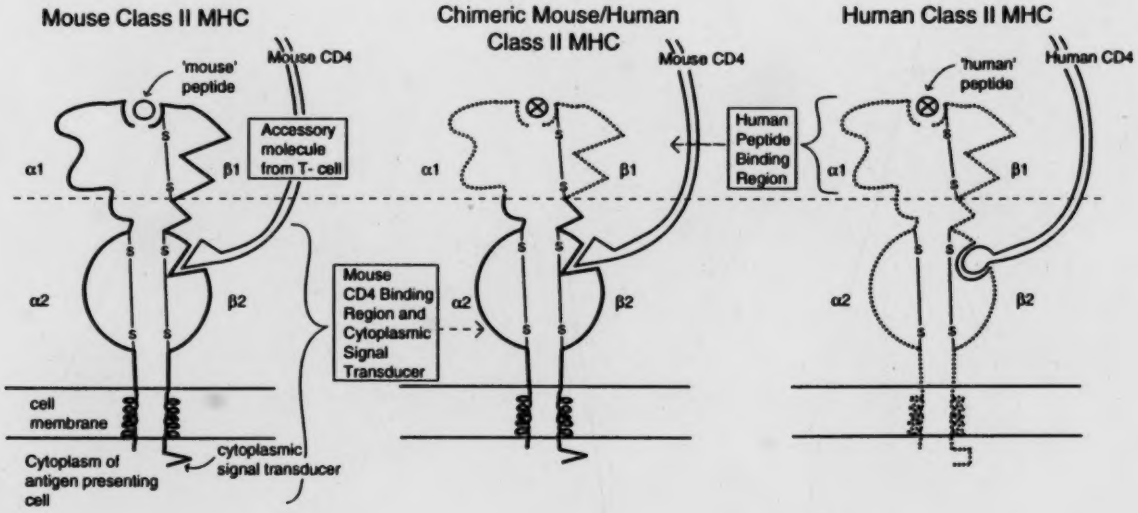



**Figure 3.1.** Schematic Diagram of mouse, human, and mouse/human chimeric MHC class II molecules. The chimeric MHC binds peptides restricted to human class II, but is capable of interacting with the mouse CD4 as the β2 region is murine. The appropriate signals are transduced to the mouse APC so that both activation of naive T-helper cells and thymic education of a repertoire of mouse T-cells that recognize 'human' peptides on the human MHC occurs in the transgenic animal.

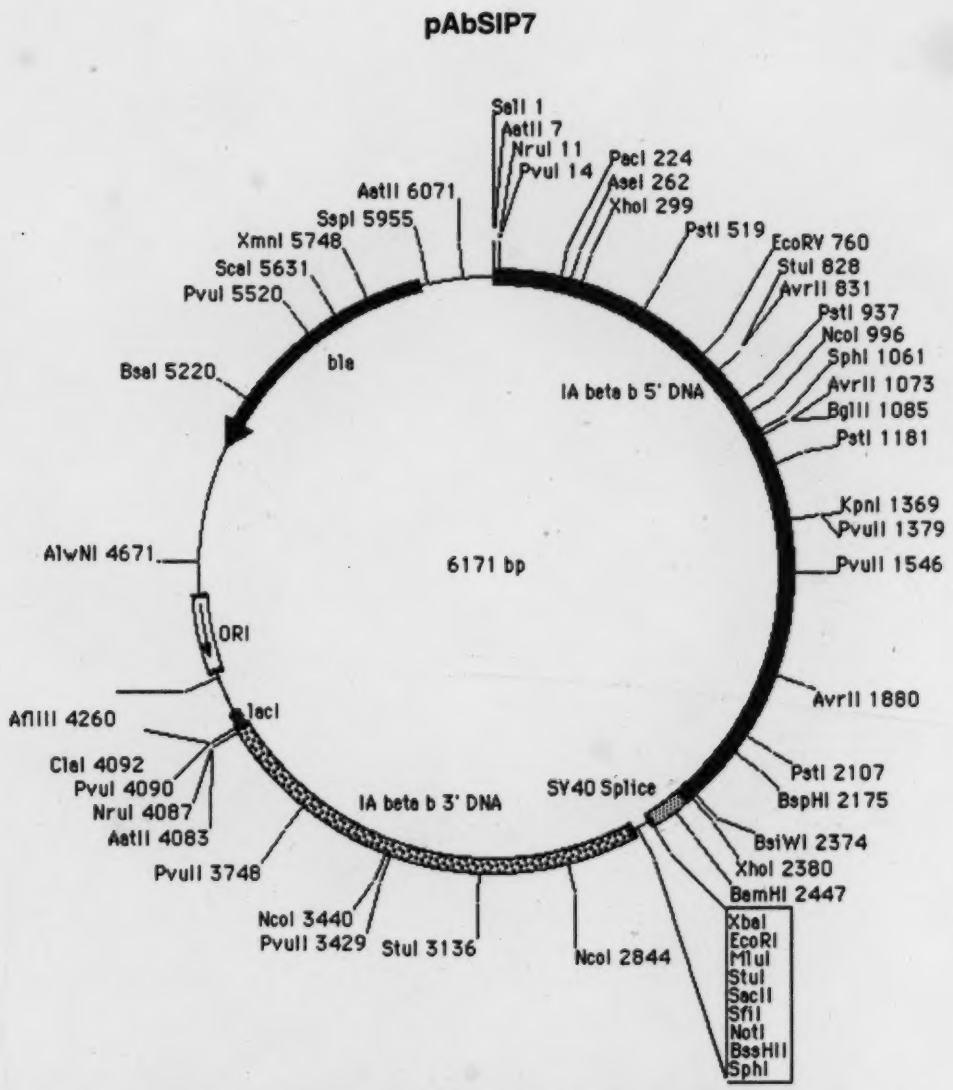



**Figure 3.2.** Schematic representation of the vector used to create chimeric transgenic mice. Chimeric mouse / human class II cDNAs were cloned into pAbSIP7 using the XbaI/NotI sites in the polylinker. Before injection into mouse embryos the plasmids were digested with AatII to remove all bacterial sequences. Inserts were purified and micro-injected into FVB embryos by Dr. Peter Dickie.

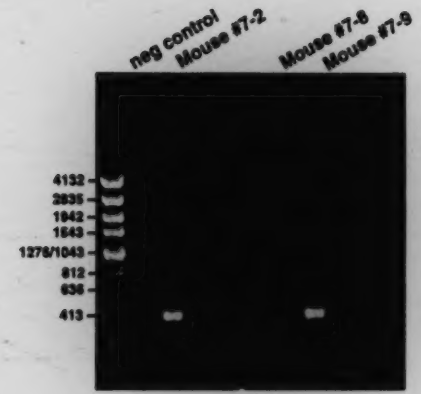



**Figure 3.3**. Identification of transgenic chimeric mice through PCR. Genomic tail DNA was purified and amplified using primers specific for the human class II α chain. Mouse # 7-2 and mouse #7-9 were positive for the transgene and mouse # 7-8 was negative. The negative control was tail DNA from a nontransgenic mouse born in a previous litter.

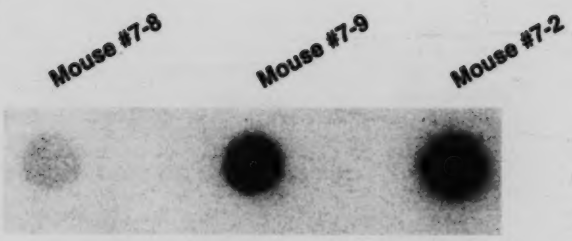



**Figure 3.4.** Dot blot analysis of chimeric transgenic mice. Tail DNA from PCR positive and negative mice was analyzed by dot blot in order to determine if the animal was homozygous for the chimeric DQ8 transgene.DNA (10μg) was applied to nitrocelluluose filters & probed with $^{32}P$ labelled probe specific for the α chain of DQ8. Mouse #7-8 was negative, mouse #7-9 was heterozygous, and mouse #7-2 was homozygous.

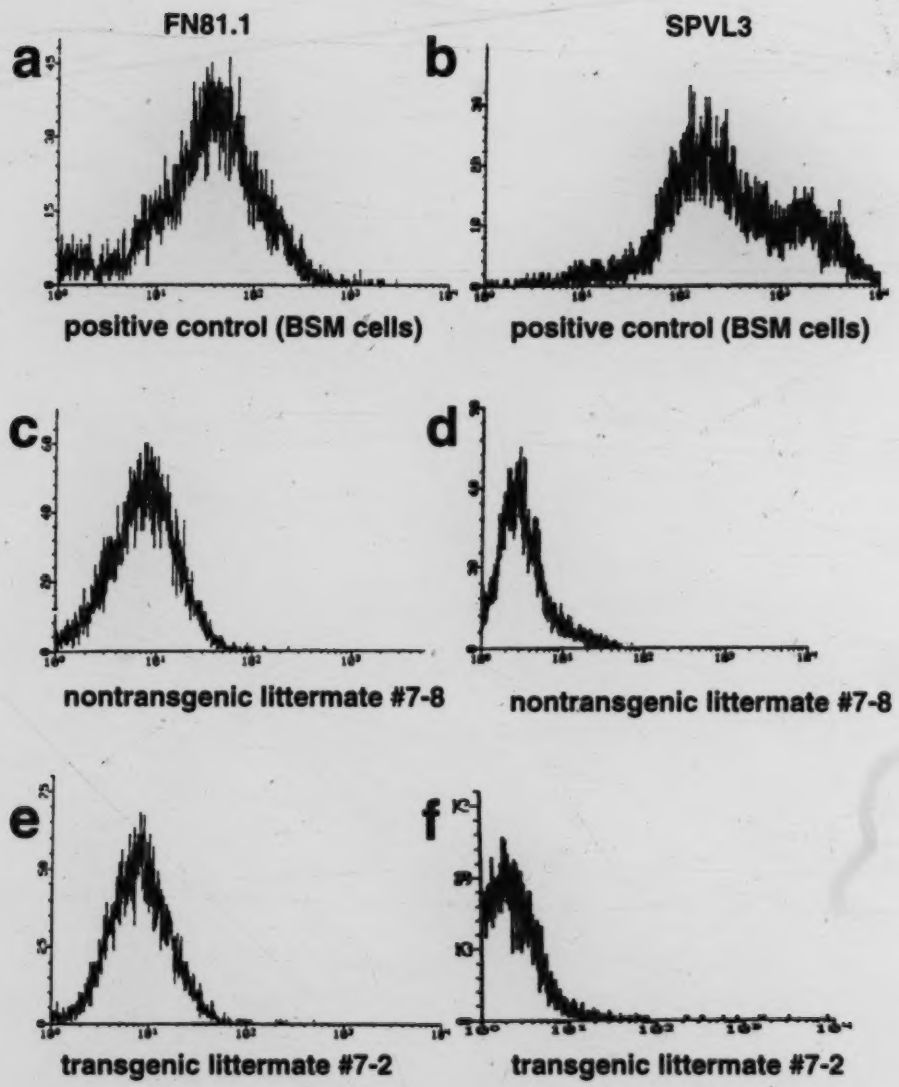



**Figure 3.5.** Analysis of chimeric mouse IA / human DQ8 expression in transgenic mice. DQ8 positive BSM typing cells (**a & b**), splenocytes from a nontransgenic littermate (**c & d**) and splenocytes from a homozygous transgenic mouse (**e & f**) were stained with the FITC conjugated monoclonal antibodies FN81.1 (**a, c, & e**) or SPVL3 (**b, d, & f**) and analyzed by flow cytometry.

# CHAPTER IV

## EXPRESSION AND PRELIMINARY EVALUATION OF FUNCTION OF HLA-DQ8 GENES IN $IA_\beta^{null}$ TRANSGENIC MICE

### INTRODUCTION

A new twist on the study of T cell epitopes to diabetic autoantigens occurred when we began a collaboration with Dr. L. Wicker of Merck Research Laboratories, Rahway, New Jersey. In 1992 Dr. Wicker created DQ8 genomic transgenic NOD/Lt mice using the DQA*0301 (H11A) and DQB*0302 (X10A) cosmids supplied by Jack Strominger (140). Upon characterization of these animals it was found that the majority of T cell responses were restricted to the endogenous IA molecules - only 1/52 T cell hybridomas from OVA were restricted to DQ8 (Linda Wicker personal communication). The most logical solution to overcome this was to remove the mouse class II from the equation. This has been done by other research groups but never in a NOD background, the animal model of diabetes (141). Dr. Tony Jevnikar from the University of Western Ontario crossed $IA_\beta^{null}$ $IE_\alpha^{null}$ mice (142) onto NOD/Lt mice. The animals were backcrossed 11 generations until the MHC was stable and class II molecules were totally absent; the mice were $IA_\beta^{-/-}$ as well as $IE^{-/-}$ (NOD mice are known to be $IE\alpha^-$) (143). In collaboration with Dr. Wicker and Dr. Jevnikar, a breeding project was started to intercross the DQ8 transgenic and NOD knockout mice. The end result was a mouse expressing DQ8 in the absence of any endogenous mouse class II; the mice were called DQ8 NOD knockouts.

The first step in determining the feasibility of using these mice to map T cell epitopes from diabetes autoantigens was to ensure that expression of the DQ8 transgene was tissue appropriate. Two color FACS analysis ($IA^{NOD}$ versus DQ8) of the original

DQ8 $IA^{NOD}$ mice from Dr. Wicker was completed. Peripheral blood, splenocytes, and thymocytes were isolated and examined for IA and DQ expression.

We then went on to characterize the DQ8 NOD knockout mice. Various populations of immune cells were compared to the original knockout mice to see if there were any changes in the cell populations between the two groups of mice, as had been noted by other groups studying similar mice (107,141). Macrophage, CD8, B cell, and CD4 subsets were identified in splenocyte and peripheral blood preparations by staining with monoclonal antibodies.

The ability of the mice to mount an immune response to several different antigens was also determined. OVA, proinsulin, and GAD antigens were emulsified in CFA and injected into the hind foot pad of the DQ8 knockout mice. Draining lymph node cells were harvested and cultured with various antigens for 24 hours, following which the culture supernatant was assayed for IL-2 as a measure of the amount of T cell stimulation which had occurred. The specificity of the responses was confirmed through blocking with monomorphic anti-DQ monoclonal antibodies.

Another interesting approach to take with the DQ8 knockout mice was to study the capacity of superantigens (sAg) to trigger T cell responses by connecting the TCR Vβ region to the DQ8 molecule present in these animals. Superantigens are characterized by their ability to activate a large proportion of T lymphocytes from many mammalian species -- as many as 20-40% of total T lymphocytes, in contrast to 0.01% of T cells which are activated by specific antigens (144). Superantigens stimulate T cells via a 'non-classical' pathway; essentially they bind outside of the peptide binding cleft of MHC class II on the APC as well as to a limited domain of the Vβ chain of the TCR (144). Superantigens are primarily bacterial toxins such as SEA, SEB or TSST-1 that can have two different effects on humans; acute responses (e.g. food poisoning caused by SEB and SEA), or chronic auto-immune related responses caused by the superantigen activation of self-reactive T cells (145). Vβ specificity of all of these sAgs has been determined in humans; SEB (Vβ

3, 12, 14, 15, 17, 20), TSST-1 (Vβ 2), and SEA (Vβ 5.3, 6.3-4, 6.9, 7.4, 9.1) (25, 191). The MHC class II isotype preference of a few sAg (e.g. streptococcal pyrogenic exotoxin C) has yet to be resolved. There are conflicting reports in the literature on the binding of superantigens to DQ molecules. In a study using EBV typing cells, Hermann et al were only able to see binding to DQ by SEC-2 (146). This opposes data obtained by Mollik et al. They observed binding of SEA to mouse fibroblasts transfected with DQ (147). Hermann argued that the discrepancy could be for two reasons. First, weak binding to the EBV typing cells could have been masked by other HLA molecules that are strong binders to superantigens -- but this could not be the case in Mollik's experiments because the fibroblasts expressed only one HLA type. This explanation also seems unlikely for the EBV cells since monoclonal antibodies were used to pre-block other HLA types (e.g. DR) thereby removing strong binders. The second hypothesis put forward by Hermann was that there may be differences in the post translational modifications of HLA molecules in mouse fibroblasts that make them more susceptible to SEA binding. The $IA_{\beta}^{-/-}$ $DQ8^{+/+}$ mice represent a useful model to clarify this discrepancy. The mice express only one HLA type and the post translation modifications should resemble those found in humans, since the molecules are expressed in a tissue appropriate manner. Therefore, the ability of superantigens to stimulate T cells from a splenocyte population was analyzed.

## RESULTS AND DISCUSSION

Tissue appropriate expression of the DQ8 transgene was analyzed in the original $IA^{NOD}$ DQ8 mice (figure 4.1). Splenocytes, thymocytes, and PBLs were stained with two monoclonal antibodies, FITC conjugated 10-3-6 for $IA^{NOD}$ and biotin conjugated FN81.1 plus streptavidin PE for DQ8, and 10 000 events analyzed by FACScan. For the PBL analysis a gate was set that collected the entire lymphocyte population; 6% stained positive for both class II. Figure 4.1 shows 14% of the cells staining with both monoclonal antibodies in the thymus. In the spleen, approximately 50% of the cell

population stained positive for both class II markers (this agrees with the B220 staining represented in figure 4.4 h where 51% of the splenocytes stained positive for the B cell marker). There is a small population staining positive for FN81.1 and negative 10-3.6 in all three FACS profiles; 6% PBLs, 5% thymocytes, 10% splenocytes. There are two possible explanations for this result: 1) perhaps the expression of DQ8 is occurring in cells other than classical APCs, or 2) because FN81.1 was biotinylated, the signal for FL2 on the FACScan might have been be augmented by the streptavidin PE. To test the second hypothesis another set of experiments was completed whereby the same cell populations were isolated and stained with FITC FN81.1 and biotin 10-3.6 and streptavidin PE (FL2) (the opposite to above). The results were essentially the same (data not shown) in that the number of cells staining positive for FL2 and negative for FL1 was 4%, 6%, and 9% for PBLs, thymocytes, and splenocytes respectively (data not shown). Therefore it appears that these results were obtained because the biotin/streptavidin complex was simply giving a stronger signal than FITC rather than being the result of significant tissue inappropriate expression of the DQ8. Thus, in general it appears that expression of the DQ8 transgene was tissue appropriate although these experiments do not rule out the possibility that very minor populations of cells were either expressing DQ8 when they would not ordinarily express class II, and/or failing to express DQ8 when they would have ordinarily expressed class II.

The breeding project to intercross $IA_{\beta}^{-/-}$ NOD mice onto $IA_{\beta}^{+/+}$ / $DQ8^{+/+}$ NOD mice was one of the major undertaking of this thesis. Upon arrival at the University of Alberta animal facility in March 1996, both sets of mice were quarantined for about three months (e.g. until offspring born in quarantine could be serologically tested at six weeks of age). Intercross breeding was initiated while in quarantine but presumably due to the stressful environment, it was not until they were moved to conventional animals facilities that several litters were born. Figure 4.2 provides a summary of that breeding as well as subsequent breeding to derive the DQ8 NOD knockout line. On March 27, 1996 the

original animals were put together. The F1 generation did not begin to breed until July 15, 1996. At this time three breeding pairs were established. The F2 generation born to these parents were initially tested for $IA^{NOD}$ expression by staining the PBL population isolated from heparinized blood collected from the tail. Animals that were determined to be $IA^{null}$ were then tested for DQ8 status through dot blot analysis of tail DNA (figure 4.3a). Homozygote animals were twice as bright as heterozygotes due to the fact they have two copies of the transgene available to bind the $^{32}P$ probe. In order to simplify determination of DQ8 status for future offspring, staining of cell surface DQ8 expression was optimized so that the difference between heterozygotes and homozygotes could be visualized by FACScan. Animals that had two copies of the transgene stained roughly an order of magnitude brighter with 1:2000 dilution of ~1.3mg/ml FITC FN81.1 (figure 4.3b). The lineage from this point on in the pedigree was the result of a brother and sister mating; mouse #10 (male $IA_\beta^{-/-}DQ8^{+/+}$) and mouse #11 (female $IA_\beta^{-/-}$ $DQ8^{+/0}$). Because of the intercross between DQ8 homozygotes and hemizygotes, the numbers obtained in the F3 generation at first glance appear confusing. To summarize this part of the project, it is sufficient to say that a brother and sister numbered #1 and #2 were used as the founders for all present breeding and are the originators of all animals used in the experiments described in this thesis. Many of the other animals that arose from the F3 generation were used in preliminary experiments, including the generation of T cell hybridomas, which are currently under investigation in our laboratory.

Once a stable colony of $IA_\beta^{-/-}DQ8^{+/+}$ (DQ8 NOD knockout) animals was established, representative animals were sacrificed and various populations of cells characterized using FACScan. These were compared to the original knockout mice in order to determine if their immunological make-up had changed by the addition of the DQ8 molecule(figure 4.4). Splenocytes were examined using cell surface markers for the following cell populations: T helper cells (CD4), cytotoxic T cells (CD8), macrophages (F4/80), and B cells (B220) (figure 4.4 a-h). It was determined that the cytotoxic T cell,

macrophage, and B cell populations had not changed substantially upon the addition of DQ8 to a NOD class II knockout mouse. However, the CD4 T helper population increased from 6.5% to 21% indicating that some of the function immune interactions between CD4 T cells and class II molecules on APCs must have been restored through the addition of the DQ8 transgene -- particularly thymic positive selection and education. PBL populations were also analyzed using monoclonal antibodies for the CD8 and CD4 makers as shown in figure 4.4 i-l. Again the number of cytotoxic T cells was very similar between the NOD knockout and the DQ8 NOD knockout mice, whereas the T helper population increased significantly (going from 1% to 111%). This is consistent with reports on similar mice from other research groups (107,141).

The ability of the mice to mount an immune response to various antigens was evaluated. There are two popular methods to measure the amount of T cell stimulation -- IL-2 release and $^{3}H$ thymidine incorporation into DNA (reflects T cell proliferation). Both methodologies were employed in this experiment. Mice were immunized with OVA, proinsulin, or GAD. Briefly a 1mg/ml of each individual protein in PBS was mixed with an equal volume of CFA and approximately 50µl injected into the hind foot pad of the NOD knockout or DQ8 NOD knockout mice. This resulted in 25µg of protein per injection. The popliteal lymph nodes were collected 10 days later and the diameter measured. The lymph nodes of NOD class II knockout mice were very small (Table 4.1) essentially were the same size as unimmunized lymph nodes. This was expected, since a significant as an immune response would be unlikely when only 1% of PBLs were CD4 cells. In contrast, the draining lymph node diameter of the DQ8 NOD knockout mice approached that of the immunized wild type NOD/Lt mice (table 4.1). Because in practice only an insignificant number of lymphocytes could be recovered from the draining lymph nodes of the NOD knockout mice (given their small size), these animals were not analyzed further. For the DQ8 NOD knockout mice, draining lymph node cells were purified as described in the Materials and Methods. The lymphocytes were incubated with various

concentrations of protein in 96 well plates and the IL-2 accumulated in 75µl of supernatant was measured after 24 hours by ELISA. The results are shown in figure 4.5a. The OVA responses were reasonable with the IL-2 concentration reaching 6.3U/ml when 1.2µM of OVA was added. This was well above background. The results for GAD and proinsulin were less convincing. Background (T cells only no antigen) was lower than experimental wells for both of these proteins; however, the IL-2 concentrations were not very remarkable, even though they were still within the sensitivity of the assay as shown by the standard cure in figure 4.5 b. Duplicate wells of the above were established at the same time in order measure T cell stimulation using $^{3}H$ thymidine uptake. Cells were incubated for 72 hours, $^{3}H$-methyl thymidine added, and then incubated for a further 18 hours before harvesting. The result shown in figure 4.5c are the calculated stimulation index (SI) for each protein. This data mimics the IL-2 data in that the OVA results show some immune response but again the GAD and proinsulin responses are not much above background, although proinsulin was still greater than GAD as seen in the IL-2 data. SIs of less than three are generally considered to be not significant (128); in keeping with this rule the results for proinsulin and GAD are essentially negative.

In order to ensure the immune response observed to OVA was due to DQ8, we performed blocking studies. IL-2 release and $^{3}H$ thymidine uptake was again measured but this time SPVL3 (117) was used to block the DQ8 responses. Con A was used as the positive control, as stimulation of T cells with Con A would not be blocked by monoclonal antibodies to class II. These results are shown in figure 4.6 a. IL-2 values in the Con A wells was 55U/ml and antibody did not decrease this response. Responses in wells with whole OVA or OVA peptide did decrease substantially upon the addition of antibody. The proliferation data indicate the same results as the IL-2 release (figure 4.6 b). One short coming of this experiment was that an isotype matched antibody control was not added in order to ensure the effect was in fact due to blocking of DQ8 and not simply due to the presence of another variable in the experiment; this is unlikely as the Con A IL-2

concentration did not decrease upon the addition of SPVL3. Another blocking experiment that will be completed as the mice are further characterized is the blocking of the T cells through the CD4 molecule using a anti-mouse CD4 monoclonal antibody, GK1.5. This will prove that the IL-2 produced is due to the TCR on mouse T helper cells interacting with human DQ8 on mouse APCs.

The last experiment done in the initial characterization of the DQ8 NOD knockout mice was superantigen stimulation of T cells. Splenocytes were collected and stimulated for 48 hours in the presence of varying concentrations of SEA, SEB, or TSST-1 (figure 4.7). IL-2 was detected for all three superantigens. The response to TSST-1 was linear and was continuing to rise even at 10μg/ml (the highest concentration tested). The response to SEB increased linearly between 0.01μg/ml and 1μg/ml but was declining at 10μg/ml. Finally, the response to SEA reached its maximum value at 0.1μg/ml and then leveled off at higher concentrations. The data indicates that DQ8 does bind all three superantigens. Smaller amounts of SEA needed to see a maximal T cell stimulation suggests that SEA binds most strongly to the DQ8 and TCR. Because large amounts of IL-2 were not seen until higher concentrations of TSST-1 and SEB were used, it can be concluded that these superantigens do not crosslink the class II and TCR as strongly.

## FUTURE DIRECTIONS

There are many experiments that are yet to be done on these animals. In order to get a complete look at their immune system the Vβ usage in the T cell population of these mice could be studied using monoclonal antibodies. The Vβ population could then be compared to the original knockout mice in order to define the increase in the CD4 population that was observed. Also immunohistochemical staining of the thymus should be done. If DQ8 expression in the thymus is appropriate as the above FACS profiles suggest, staining of DQ8 should be evident as stippled in the cortex and confluent in the medulla. This reflects class II expression on epithelial cells in the cortex and on dendritic

cells, macrophages, and epithelial cells in the medulla (148). Currently the DQ8 NOD knockout mice are being transferred to a 'clean' animal room. The intention is to remove any environmental immune stimuli that may tend to prevent the appearance of diabetes. The NOD/Lt wild type mice are notorious for their incidence of diabetes but the NOD knockout mice do not develop diabetes at all. The DQ8 NOD knockouts will be observed to study if the introduction of the DQ8 gene increases the NOD knockout susceptibility to IDDM. Animals will be monitored both by measuring blood glucose levels and looking for the presence of insulitis in histological sections of pancreatic tissue.

To confirm the superantigen is binding to the MHC class II, monoclonal antibodies to DQ8 could be used to block the binding like was completed with the OVA stimulations.

Also the mice can be used to study the formation of trans-heterodimers between murine and human class II molecule as several groups have reported this occurring in transgenic animals (149-152). Trans-heterodimer formation would be the result of $IA_{\alpha}^{b}$ pairing with the DQB1*0302.

Before the mapping of T cell epitopes of diabetic autoantigens can begin, the amount of antigen and number of cells in the stimulation assay must be systematically titrated in order to optimize the amount of IL-2 produced (e.g. optimize the stimulation). This will make positive results more obvious. One of the simplest things to try is injecting a larger amount of proinsulin and GAD. David et al. have used as much as 100μg of antigen in foot pad injections in order to see an immune response in their DQ8 transgenic mice (we used only 25μg) (107,153).

Even though David et al. were able to see transgenic DQ8 (genomic) $IA^{null}$ mice responding to antigen, this remains an area of potential disagreement given our initial low responses to GAD and proinsulin. Because of this two strategies should be employed for the mapping of T cell epitopes to GAD and proinsulin (106,107). All of the antigens used by David to generate an immune response were highly immunogenic e.g. Dermatophagoides pteronyssinus (Der p) dust mite allergen, or Trichinella spiralis. Other

groups report immune responses only when peptides were used (106,148). This will require further investigation as our mice did not give a good immune response in preliminary stimulations with GAD and proinsulin but did with OVA which is understood to be highly immunogenic. A possible approach that can be followed in order to increase the immune response of our mice is to inject the mice with pools of peptide followed by recall with individual peptides (as David has done in looking at proinsulin epitopes in DQ8 knockout mice; as there are verbal reports that his mice do not mount an immune response to whole proinsulin).

The fact that both GAD and proinsulin were recombinant proteins that lack quaternary structure because they were made in E. coli cannot be ignored. Richter et al derived a panel of seven monoclonal antibodies from a diabetic patient. All were specific for GAD65 (154). Using deletion mutant analysis it has been discovered that six of the seven monoclonal antibodies recognize conformational epitopes in the middle and C terminal regions of GAD. Because the humoral epitopes depend on quaternary structure the B cells will be unable to efficiently capture the antigen and therefore, processing and presentation of peptides will also be inefficient.

From the data presented it can be concluded that the DQ8 NOD knockout mice will prove to be useful in the study of immunology. Their success in the original project, the mapping of T cell epitopes prevalent in diabetes, has yet to be determined; however, the superantigen stimulation data is promising in that the mice will prove to be useful in elucidating superantigen binding patterns to DQ8.

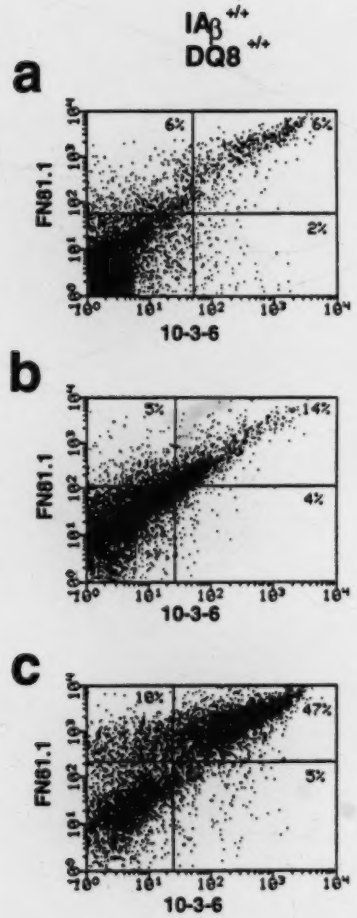



**Figure 4.1.** Expression of the HLA-DQ8 transgene versus endogenous $IA^{NOD}$ in the original $IA^{NOD}$DQ8 transgenic mice. PBLs (**a**), thymocytes (**b**), and splenocytes (**c**) were isolated from a $DQ8^{+/+}$NOD/Lt mouse. Cells were double stained with FITC conjugated 10-3.6 (anti-$IA^{NOD}$) and biotin conjugated FN81.1 (anti-DQ8) plus streptavidin PE and analyzed by FACS.

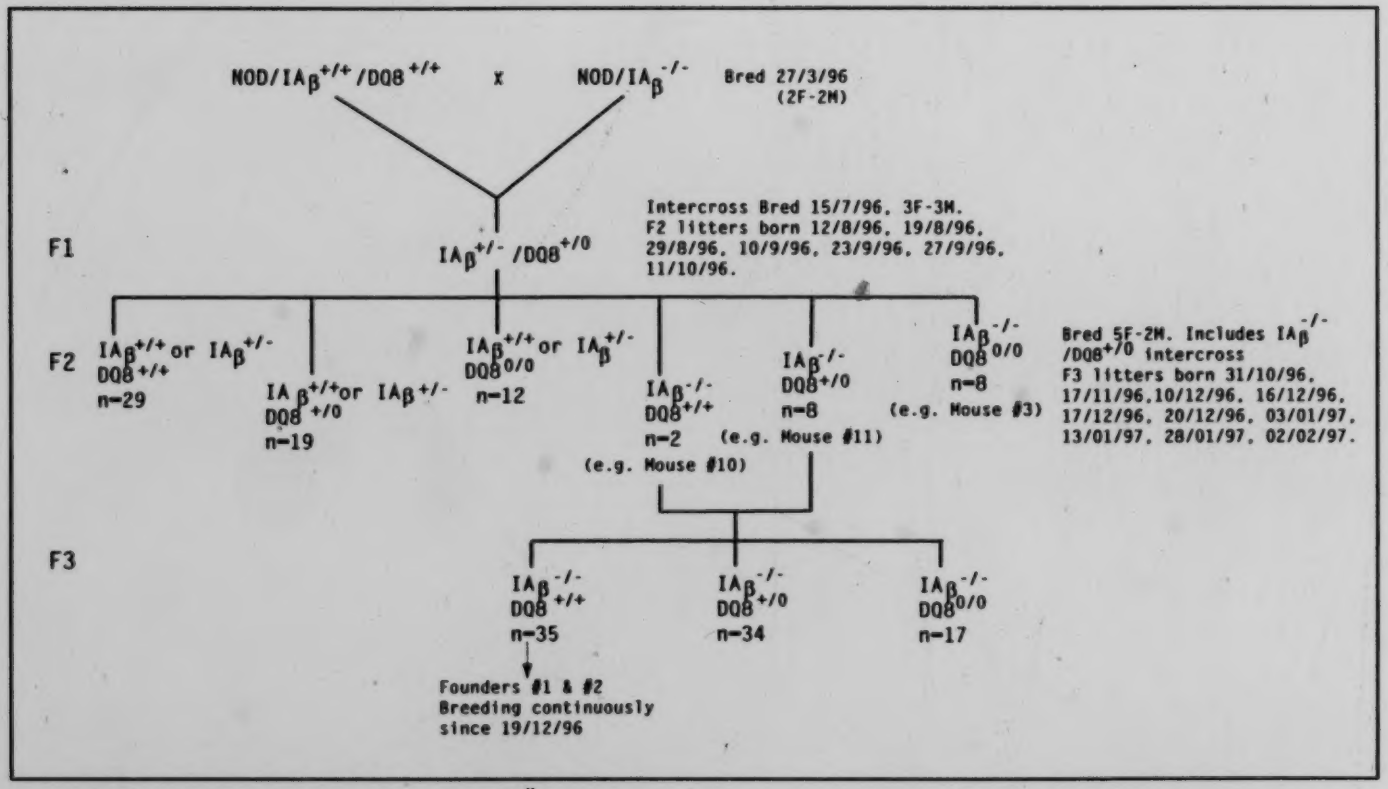

NOD/IAβ+/+/DQ8+/+ x NOD/IAβ-/- Bred 27/3/96 (2F-2M)
F1
IAβ+/-/DQ8+/0
Intercross Bred 15/7/96, 3F-3M. F2 litters born 12/8/96, 19/8/96, 29/8/96, 10/9/96, 23/9/96, 27/9/96, 11/10/96.
F2
IAβ+/+ or IAβ+/- DQ8+/+ n=29
IAβ+/+ or IAβ+/- DQ8+/0 n=19
IAβ+/+ or IAβ+/- DQ80/0 n=12
IAβ-/- DQ8+/+ n=2 (e.g. Mouse #10)
IAβ-/- DQ8+/0 n=8 (e.g. Mouse #11)
IAβ-/- DQ80/0 n=8 (e.g. Mouse #3)
Bred 5F-2M. Includes IAβ-/- /DQ8+/0 intercross F3 litters born 31/10/96, 17/11/96, 10/12/96, 16/12/96, 17/12/96, 20/12/96, 03/01/97, 13/01/97, 28/01/97, 02/02/97.
F3
IAβ-/- DQ8+/+ n=35
IAβ-/- DQ8+/0 n=34
IAβ-/- DQ80/0 n=17
Founders #1 & #2 Breeding continuously since 19/12/96

**Figure 4.2.** Generation of HLA-DQ8 $^{+}$, IA$_{\beta}^{null}$ NOD/Lt Mice. Presence of the HLA-DQ8 transgene was monitored by FACS analysis of PBL populations using the monoclonal antibody FN81.1. Presence of the IA$_{\beta}$ gene was evaluated by FACS analysis of PBLs using the monoclonal anitbody 10-3.6. Founders #1 & #2 both originated from the cross between #10 (M) and #11 (F). All mice in the line and used in the experiments described in this thesis are from these two founders.

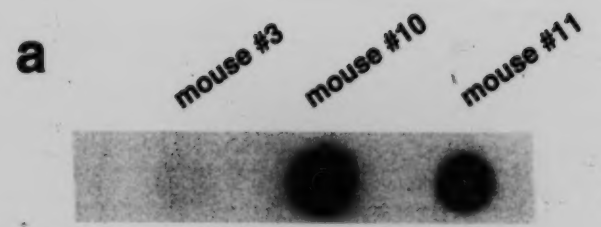
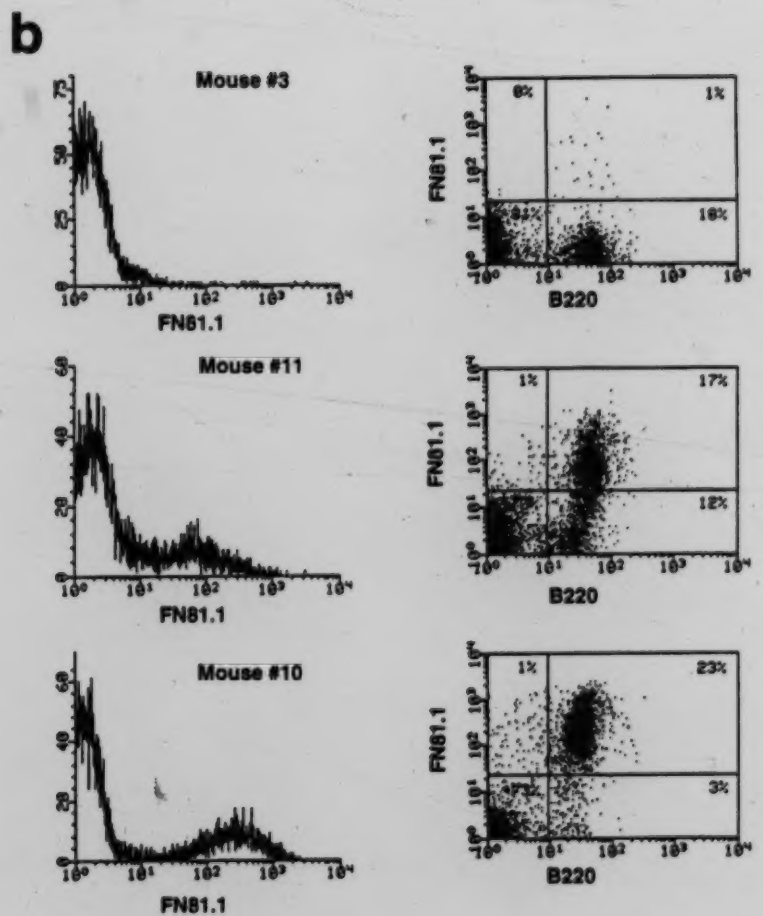
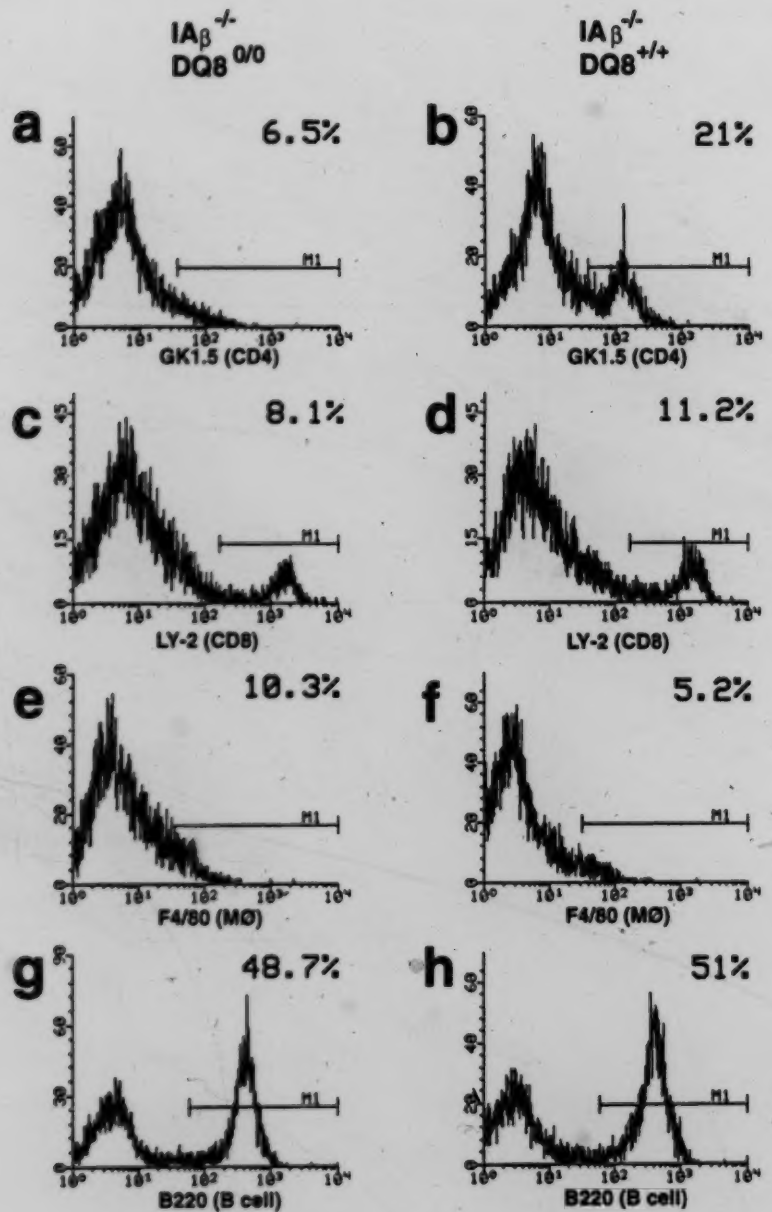



**Figure 4.3.** Determination of HLA-DQ8 genotype by dot blot analysis. DNA was prepared from tail digests as described in the Materials and Methods. DNA (10μg) was applied to a nitrocellulose filter and probed with a $^{32}$P labelled probe specific for DQ8 (**a**). Mouse #3 is negative, mouse #10 is homozygous, and mouse #11 is heterozygous. This was then confirmed through FACS analysis of PBLs from these animals (**b**). PBLs were purified and stained with FN81.1 as previously described.


b
Mouse #3
FN81.1
FN81.1
B220
Mouse #11
FN81.1
FN81.1
B220
Mouse #10
FN81.1
FN81.1
B220


IAβ -/-
DQ8 0/0
IAβ -/-
DQ8 +/+
a
6.5%
GK1.5 (CD4)
b
21%
GK1.5 (CD4)
c
8.1%
LY-2 (CD8)
d
11.2%
LY-2 (CD8)
e
10.3%
F4/80 (MØ)
f
5.2%
F4/80 (MØ)
g
48.7%
B220 (B cell)
h
51%
B220 (B cell)
M1

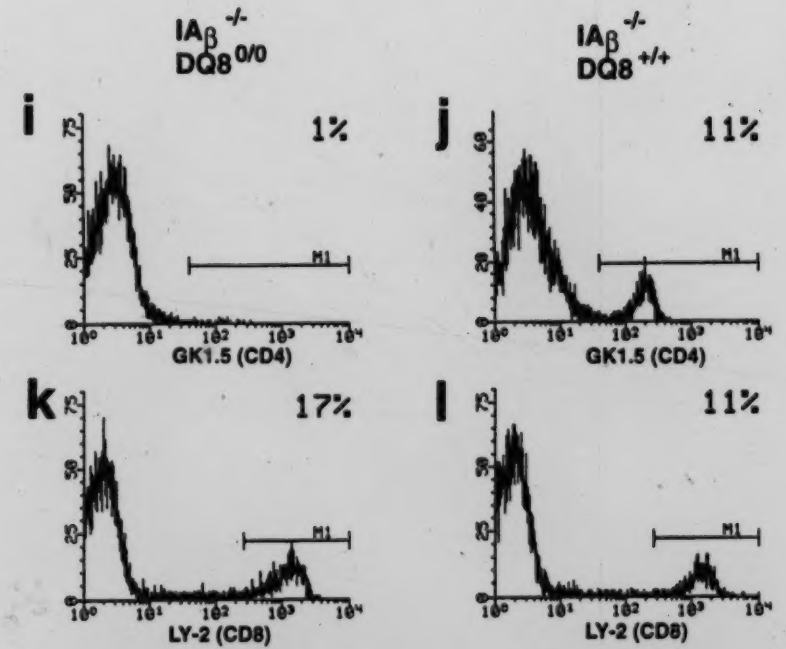


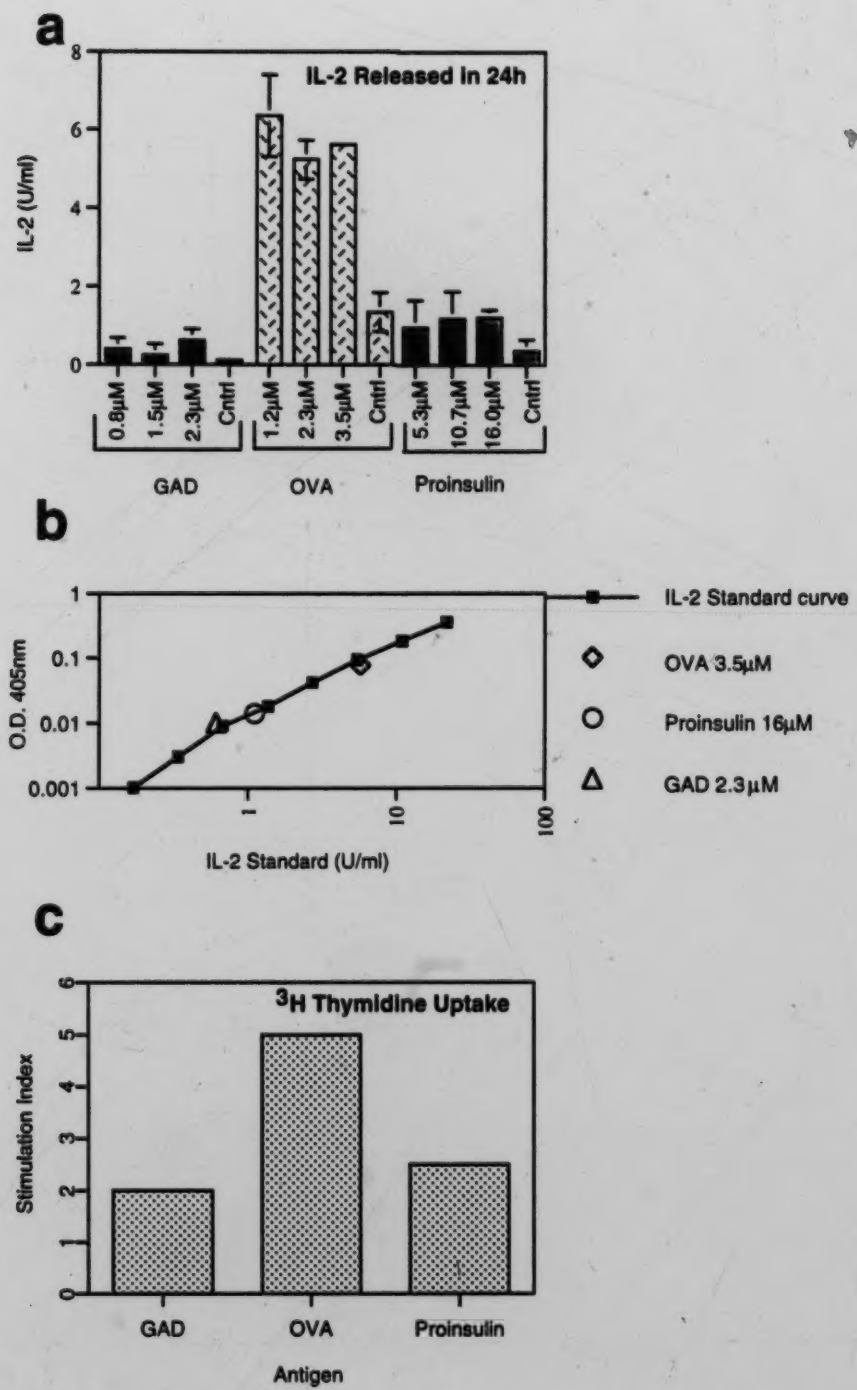

**Figure 4.4**. Comparison of various populations of immune cells found in $IA_\beta^{-/-}$ $DQ8^{+/+}$ mice versus $IA_\beta^{-/-}DQ8^{0/0}$ mice. Splenocytes (**a-h**) and PBLs (**i-l**) were purified and stained with FITC conjugated antibodies as follows: GK1.5 (**a,b,i,j**), LY-2 (**c,d,k,l**), F4/80 (**e,f**), & B220 (**g,h**). $IA_\beta^{-/-}/DQ8^{0/0}$ mice (**a,c,e,g,i,k**) were compared to $IA_\beta^{-/-}/DQ8^{+/+}$ mice (**b,d,f,h,j,l**). Portion of cells staining within the M1 gate are shown as percentages for each FACS plot.

**Table 4.1.** Measurement of draining lymph node size.

| Antigen | Lymph node diameter (mm) | |
|---|---|---|
| | $IA_\beta^{-/-}$ $DQ8^{0/0}$ | $IA_\beta^{-/-}$ $DQ8^{+/+}$ |
| OVA | 1.3±0.4 | 5.0±0.2 |
| Proinsulin | 1.5±0.3 | 3.5±0.8 |
| GAD | 1.0±0.2 | 4.3±0.7 |

$IA_\beta^{-/-}$ $DQ8^{0/0}$ and $IA_\beta^{-/-}$ /$DQ8^{+/+}$ mice were inoculated in the hind foot pad with of OVA, proinsulin, or GAD (25µg protein/50µl PBS) emulsified in an equal volume of CFA. Draining lymph nodes were isolated 10 days later and the diameter measured using a ruler. For comparison wild type NOD/Lt lymph nodes measured 5-7mm following immunization with OVA.

**Figure 4.5.** *In vitro* responses of draining lymph node cells primed with different antigens. $IA_{\beta}^{-/-}$ $DQ8^{+/+}$ mice were inoculated in the hind foot pad with OVA, GAD, or proinsulin (25µg protein/50µl PBS) emulsified in an equal volume of CFA. Popliteal lymph nodes were collected 10 days later and lymphocytes purified as described in the Materials & Methods. Cells ($3x10^5$/200µl) were incubated with various concentrations of antigen in triplicate and 24h later 75µl of the supernatants were collected and assayed for IL-2. The absorbance as measured by ELISA was read off of an IL-2 standard curve (**b**) in order to determine the amount of IL-2 released. Cntrl represents the background IL-2 produced in wells with no antigen added (e.g cells only). An identical plate was set up at the same time but cultured for 72h after which 1µCi/well $^3H$ thymidine was added. Eighteen hours later the cells were harvested. The average cpm of each triplicate and the standard error are as follows: OVA 12400±694 - background 2300±260, GAD 3006±181 background - 1504±132, proinsulin 4748±294 - background 1899±261. The stimulation indexes were also calculated (average of triplicate wells with Ag ÷ average of triplicate control wells) (**c**).


a
IL-2 Released in 24h
IL-2 (U/ml)
0
2
4
6
8
0.8μM
1.5μM
2.3μM
Cntrl
1.2μM
2.3μM
3.5μM
Cntrl
5.3μM
10.7μM
16.0μM
Cntrl
GAD
OVA
Proinsulin
b
O.D. 405nm
1
0.1
0.01
0.001
1
10
100
IL-2 Standard (U/ml)
IL-2 Standard curve
OVA 3.5μM
Proinsulin 16μM
GAD 2.3μM
c
3H Thymidine Uptake
Stimulation Index
0
1
2
3
4
5
6
GAD
OVA
Proinsulin
Antigen

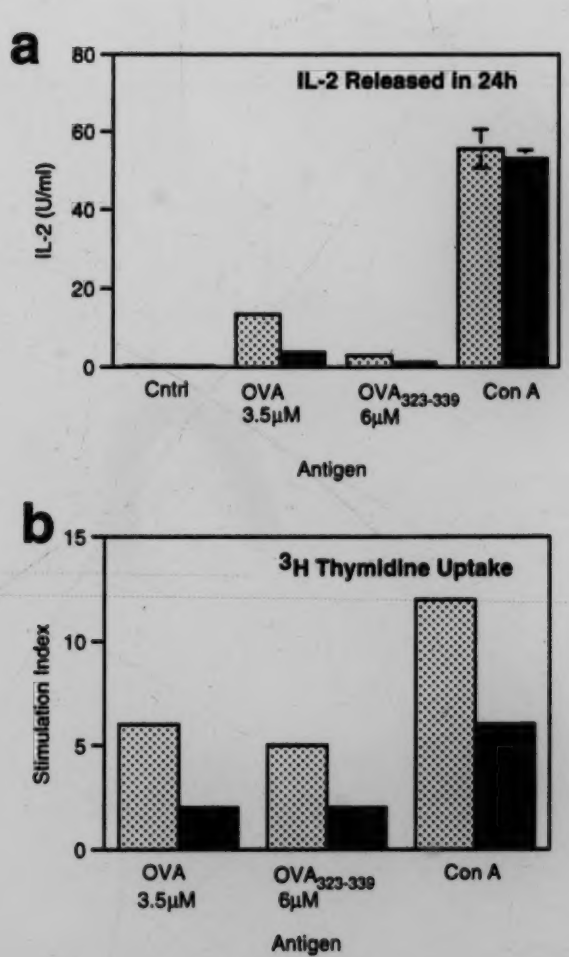



**Figure 4.6**. Blocking of DQ8 restricted responses. $IA_{\beta}^{-/-}$ $DQ8^{+/+}$ mice were inoculated in the hind foot pad with OVA as previously described. Ten days later draining lymph nodes were collected and the lymphocytes purified. Cells ($3x10^{5}$/200μl) were incubated in triplicate with Con A, whole OVA, or $OVA_{323-339}$ peptide in the absence (stippled bars) or presence (solid bars) of 10 μg/ml of the blocking monoclonal antibody SPVL3 (**a**). Duplicate cultures were also incubated for 72h, followed by the addition of 1 μCi/well $^{3}H$ thymidine and a further 18h incubation prior to harvesting. SI were calculated as previously described (**b**). The average cpm of each triplicate± the standard error is as follows: OVA (3.5 μM) 8500±434 +antibody 2800 ±189, $OVA_{323-339}$ (6μM) 7400±347 + antibody 3200 ±275, Con A 18000 ±693 +antibody 86000±399, background 1500±231.

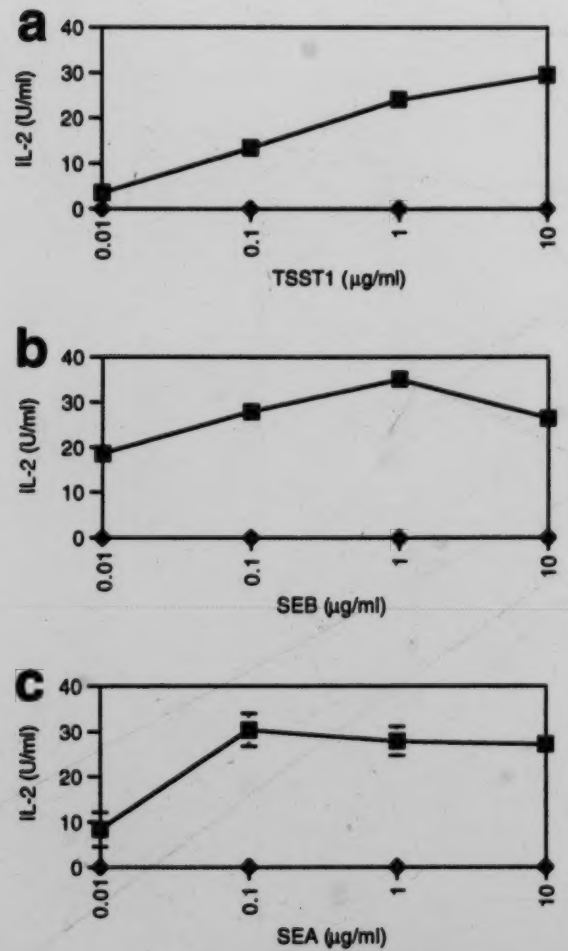



**Figure 4.7.** IL-2 released in 48 h by DQ8 transgenic splenocytes stimulated with various concentrations of 3 different super antigens. Splenocytes from $IA_\beta^{-/-}$ $DQ8^{0/0}$ mice (diamonds) and $IA_\beta^{-/-}$ $DQ8^{+/+}$ mice (squares) were recovered and incubated in triplicate with various concentrations of TSST-1 ( **a**), SEB (**b**), or SEA (**c**) for 48 h. IL-2 was measured after 48h by ELISA. Panel **d** shows the IL-2 released in 48h for the same splenocytes (closed bar $IA_\beta^{-/-}DQ8^{+/+}$; open bar $IA_\beta^{-/-}$ $DQ8^{0/0}$) incubated either with no antigen or with 1µg/ml Con A.

# CHAPTER V

# CHARACTERIZATION OF T CELL EPITOPES TO DIABETIC AUTOANTIGENS USING AN EBV EXPRESSION VECTOR

## INTRODUCTION

Epstein-Barr virus (EBV) is a human herpes virus which infects B cells and causes infectious mononucleosis (155). EBV has also been implicated as the cause of Burkitt's lymphoma, nasopharyngeal carcinoma (156), and thymic carcinoma (157).

EBV is well documented as an effective means of transforming human B-lymphocytes into immortalized cell lines (158-160). In nature EBV has a limited host range, infecting human B cells and some human nasopharyngeal cells that express the C3d complement receptor (161,162). The viral DNA is present as an episome in the nucleus of the host cell (163). Two elements are essential for EBV replication - the *cis*-acting origin for plasmid replication (*ori* P) and the *trans*--acting EBV nuclear antigen (EBNA-1) (164,165). The *ori* P is comprised of two different elements, a region of 30bp long A-T rich sequences repeated 20 times in tandem and a 125bp region containing a 65bp dyad symmetry element related to the tandem repeats (164). DNA replication is initiated near the dyad element of *ori* P and the tandem repeats serve as a replication fork barrier (166). EBNA-1, a 65-80 kDa protein, is the only nuclear antigen absolutely essential for virus replication. The tandem repeats in *ori* P are an EBNA-1 dependent enhancer that plays a role in both replication and transcription (165).

These features of EBV have been utilized to create eukaryotic expression vectors (121). Such vectors contain *ori* P, EBNA-1, and a resistance marker, and they have proven effective for expressing proteins in eukaryotic cells. Recently a commercial vector,

pREP4 (Invitrogen, San Diego, CA), containing thyroid peroxidase (TPO) cDNA was transfected into autologous EBV transformed B cells in order to stimulate TPO-specific T cell clones isolated from a patient with Grave's disease (167). This system served as a model for the experiment described below.

As previously mentioned, the overall goal of this project was to identify T cell epitopes in diabetes associated islet cell autoantigens. The hypothesis was that if the entire autoantigen cDNA was transfected into an APC, some portion of it would make its way to the class II compartment and the peptides created from it would be transported to the cell surface bound to the class II molecule. The transfected APC could then be used to trigger T cell hybridomas created against the autoantigen. From there stable transfectants expressing subsegments of the autoantigen cDNA could be made and used to narrow down the regions of the protein that contained epitopes recognized by the T cell hybridoma. This approach would in theory decrease the number of overlapping peptides that ultimately would need to be made, and thus would be particularly attractive for mapping T cell epitopes in large proteins such as GAD.

The plasmid which we created for this project was named pDLEBNA. It contained all of the traditional elements found in any EBV expression vector, as well as a few unique features. The bacterial origin of replication was pUC based, giving the plasmid a higher copy number per bacterial cell. This was advantageous when large quantities of purified plasmid DNA were required. pDLEBNA utilized the Srα promoter to drive expression of the cloned cDNA insert (e.g. GAD)(168). This promoter contains both the early and late promoters from SV40 as well as the SV40 origin. We anticipated that this arrangement of SRα would give higher levels of transcription for two reasons: 1.) the powerful SRα promoter was driving expression of the inserted gene, and 2.) more EBNA-1 would lead to a higher gene copy number, as it has been suggested that the more EBNA-1 in the cell, the higher the expression of the gene of interest due to more episomal replication (169).

Reports in the literature suggest that often endogenously expressed proteins need 'help' in order to be targeted into the class II compartment (170,171). In order to ensure that our experiments did not fail due to the lack of such 'help', in one version of our vector (pDLEBNA-Ii), the N-terminal signal sequence of the human invariant chain (Ii) which is responsible for targeting to the class II compartment, was cloned and inserted into pDLEBNA upstream of the multiple cloning site. Ii is a type II transmembrane protein (the N terminus is located in the cytosol and the C terminus in the ER). This Ii version of the plasmid was expected function the same as the original pDLEBNA except that the protein translated in the B cell would consist of a fusion between the Ii signal (N-terminus) and the cloned autoantigen.

To test the system while transgenic mice were being generated, two T cell hybridomas made previously in other transgenic animals, were acquired from our collaborator Dr. Linda Wicker (Merck, Inc. Rahway, NJ). The first of these recognized the peptide OVA323-339 bound to DQ8 (L. Wicker unpublished) and is called 47.28. The second hybridoma, created from chimeric DR4 transgenic mice (104), recognized the GAD273-285 peptide presented on DR4 and is called 33.1 (83). Both hybridomas were generated by inoculating the transgenic mice with the mentioned peptides emulsified in CFA. In this series of experiments, *in vitro* IL-2 production by the OVA and GAD T cell hybridoma induced by using the transfected BSM cells as APC's was the measurement used to ensure the vector was functioning. The corresponding peptides added into the assay provided the positive controls. The IL-2 produced was measured directly by ELISA, and Western blots were done to see if the expected protein (OVA or GAD) could be detected inside the transfected B cell lines.

## RESULTS AND DISCUSSION

The resistance marker used for selection was the hygromycin-phosphotransferase

gene (hph). The gene was obtained using PCR from the original EBO-pcD-Leu2 vector of Bob Margolskee (121) to create the restriction sites SpeI and MfeI on the 5' and 3' ends respectively (primers were TCTCTCACTAGTCCACCATGAAAAAGCCTGAACTCACC and CTCTCTCAATTGTCTATTCCTTTGCCCTCGGA). This product was ligated into the XbaI and EcoRI sites of the expression vector pEF1αBSD7. The XbaI / SpeI and MfeI / EcoRI combinations have identical overhangs so that they anneal but the original restriction sites are not recreated after ligation (figure 5.0) [for details on ligations see Materials and Methods]. The EBNAoriP portion of the vector was obtained from Bob Margolskee's vector EBOpLPP (172). This 4920bp fragment of DNA was excised using BamHI and ClaI and force cloned into the same sites of pBluescript KS- (Stratagene La Jolla, CA). In separate reactions, to each end of the EBNAoriP segment a linker was added in order to change the restriction sites. At the 5' end the BamHI site was opened and then destroyed -- 5'SalI-ClaI3' sites were added by ligating in a linker (linker 1) consisting of oligonucleotides (GATCATCGATGTGGTCGAC and GATCGTCGACCACATCGAT). The 3' end of EBNA*oriP* was treated in a similar manner; a linker (linker 2) was added into the Cla I site resulting in 5'XhoI-PstI-HindIII3' sites using the sequence CGAAGCTTCTGCAGCTCGAGA and CGCTCGAGCTGCAGAAGCTT (figure 5.0c). In a ligation utilizing the unique MluI site in the *ori*P, the EBNA*ori*P was reassembled so that both linkers were present. Each plasmid comprised of linker 1 or 2 was digested with XbaI (from the pBluescript polylinker upstream of the 5' linker 1) and MluI. The vector backbone containing linker 2, EBNA-1, and 1/2 of the *ori*P was ligated to the 1322bp insert containing the remainder of the *ori*P attached to linker 1. The result is depicted in figure 5.0c. Finally pDLEBNA was assembled by ligating the 4920bp EBNA*ori*P ClaI - HindIII fragment, the 2829bp EF1αhph ClaI - SalI fragment, and the SrαSD7 (83) 3176bp SalI - Hind III fragments together as shown in figure 5.0 and 5.1. The resultant vector had human elongation factor 1 alpha enhancer promoter driving expression of the hph gene, the SV40 late promoter

transcribing the EBNA-1 and the high level SRα SV40 early promoter driving the cDNA insert (figure 5.1). The resulting mini preps [Materials and Methods] were mapped with Mlu I/EcoRI, PvuII/SalI, and XhoI to ensure the vector was assembled properly (figure 5.3).

The pDLEBNA-Ii version of the vector was then constructed. The endosomal targeting sequence of human invariant chain was isolated through RT-PCR of WT 49 (Human Mutant Cell Repository GM03098A) mRNA. The primers were designed with a SpeI site on the 5' end and 5'XbaI-EcoRI-MluI3' sites on the 3' end (primers - ÀCTAGTCCACCATGGATGACCAGCGCGACCT and ACGCGTGAATTCTCTAGACATGCGCAGGTTCTCCAGCT). The resulting cDNA (237bp) fragment encoded the first 79 AA of what is commonly known as the p31 isoform. The difference between the truncated p31 isoform and the full length p33 isoform is the first 15 AA of the Ii ORF are present in p33 but absent in p31. Studies have shown that the p33 isoform is retained in the endoplasmic reticulum and thus we removed the residues that might cause our fusion proteins to be retained in the ER (22). Our translated protein begins at the second ATG codon in the Ii cDNA sequence; this provides the signal sequences necessary for endosomal targeting (23) as the 79 AA proteins present are located in the transmembrane region of Ii . Our protein ends just before the CLIP region which is located between aa 83 and 107 (18). This is the region of Ii found in the peptide binding groove of class II. The 237bp Ii fragment was inserted into the pDLEBNA vector using the XbaI - MluI sites in the polylinker (figure 5.2). The XbaI site on the 5' end was destroyed but recreated by the PCR primers on the 3' end to allow autoantigens to be cloned in using the enzymes XbaI/NotI. A PvuII site located within the Ii sequence was critical to the mapping, as digesting with PvuII and SalI created an extra 376 bp band in pDLEBNA-Ii to help distinguish it from pDLEBNA (figure 5.3). Present between the signal sequence and the gene of interest were 5'XbaI, EcoRI, and MluI 3'

sites. These restriction enzymes have 6 bp recognition sequences and were designed so that no matter which of them were used to clone, the autoantigen would remain in frame during translation. In our case, because XbaI was used to clone, the EcoRI and MluI sites are removed and serinine and arginine bridged the Ii signal sequence and the cloned antigen (figure 5.2).

The GFP (primers - TCTAGACCACCATGAGTAAAGGAGAAGAACTTTTC and, GCGGCCGCTATTATTTGTATAGTTCATCCATGCC), OVA (primers - CTCTAGAATGGTATACCTGGGTGCA and GCGGCCGCTCTGGGTCTTGTTGGAA) and GAD (primers - TCTAGAATGGCATCTCCGGGCTCTG and GCGGCCGCTTATAAATCTTGTCCAAGGCGTT) minimal coding regions were created by PCR and cloned into pDLEBNA, pDLEBNA-Ii, and EBOpLPP using the XbaI / NotI sites from the polylinker .

The pDLEBNA construct was tested for function by using the GFP gene as a reporter. This allowed for protein expression to be monitored as the GFP emits fluorescence at 508nm; a wavelength measurable using FL1 of the FACS analyzer (figure 5.4). Forty eight hour expression looked promising; however, GFP expression after two weeks showed a slight decrease but was still well above the empty vector control transfected cells. For comparison, transient transfections of GFP in COS cells showed a shift in fluorescence intensity that is over $10^4$ (Dan Denney personal communication). Therefore, although our construct pDLEBNAGFP was expressing, the amount of intracellular protein produced was very low. A good positive control that is missing from this experiment is EBOpLPPGFP. Since this vector is proven in the literature it would have been good to compare with our GFP vector (6). Unfortunately this construct was not made due to a shortage of time.

Since the 33.1 and 47.28 T cell hybridomas were not generated in our laboratory, they were tested to ensure that they had maintained their TCR and had retained their specificity to the correct peptide. The presence of the TCR on both T cell hybridomas was confirmed by staining with H57-597 (hamster anti-mouse TCR) and analyzing by FACScan (data not shown). The verification of peptide specificity was accomplished by stimulating the T cells with the appropriate peptide and using BSM ($DR4^+DQ8^+$) B cells as APCs (figure 355 a&b). Both hybridomas reacted as expected and produced IL-2 levels of over 20U/ml when 10μM of peptide was added. Whole antigen with BSM cells did not trigger the hybridoma cells, and this is consistent with the fact that BSM cells are B cells which are inefficient at phagocytizing whole proteins (25). B cells internalize antigen by receptor mediated endocytosis using their surface immunoglobulins (sIg) as the receptors. After antigen binds, the sIg augments B cell function through increased signaling and concentrating antigen for subsequent internalization and processing (173). It is possible that BSM cells lack functional sIg for this to occur. Another route for antigens to enter the B cell is through the CR1 complement receptor. Antigens are opsonized by C3b which then binds to the CR1(174). If this route is important it would not be functional in our system as no complement was present ( the BSM cells were maintained in heat inactivated serum and the assays were done using Nutridoma). A third possibility is that it has been observed that it takes time for the concentrating of antigen on the B cell surface to occur (175). Perhaps the time of the assay was too short for the antigens to concentrate on the cell surface before endocytosis and processing. Prepulsing the BSM cells with antigen before adding the hybridomas into the assay could of been tried but since there was an alternative this was not done.

The alternative was to use splenocytes from a $IA\beta^{-/-}DQ8^{+/+}$ transgenic mouse as APCs. Because a portion of the splenocyte population have the ability to phagocytose antigens, they are more effective at internalizing and processing whole proteins and

presenting resulting peptides on the cell surface. Upon stimulation of 47.28 with splenocytes (figure 5.5 c), IL-2 levels in response to the peptide were close to levels achieved when PMA and Ionomycin or anti-TCR was used. IL-2 was produced in response to whole OVA confirming that the 323-339 epitope is naturally processed. Unfortunately no DR4 chimeric transgenic mice were available to test the 33.1 cells in this manner. In any case the results indicate that the hybridomas could now be used to test the pDLEBNA and pDLEBNA-Ii constructs.

Even though expression was low (as determined by the GFP analysis), it was necessary to test if endogenously expressed OVA and GAD proteins would be properly processed and the peptides expressed on the surface of the BSM cell in conjunction with the MHC class II molecules. This was accomplished by using the various transfected BSM cell lines as APCs (figure 5.6 and 5.7). OVA and GAD were also cloned into Bob Margolskee's EBOpLPP vector to use as a positive control (119,172). Varying ratios of BSM transfectants to T hybridoma cells were used. For the OVA constructs (figure 5.6) the 5:1 BSM:T cell ratio gave the best IL-2 values. Background values of IL-2 were measured by transfecting empty pDLEBNA into BSM cells (figure 5.6 a). The IL-2 ELISA measures values as low as 0.6U/ml as determined by standard graphs similar to figure 4.5b; therefore within the sensitivity of this assay no IL-2 was detected. EBOpLPP OVA, pDLEBNA OVA , and pDLEBNA-Ii OVA all gave IL-2 values that were above background, suggesting that the constructs were processing at least a portion of the expressed protein and resulting peptides were making their way to the surface in the class II molecule; however, the amount of IL-2 produced was low. Because the IL-2 concentration was approximately the same with each OVA transfectant, it can be concluded that all three constructs were functioning at the same level. Positive controls --BSM transfectants and 47.28 hybridoma cells at a 5:1 ratio with 6μM of the OVA323-339 peptide -- gave IL-2 values of 13-27 U/ml, ~5 fold higher than the transfected cells. Experiments

with the GAD constructs were completed in the same manner (figure 5.7). IL-2 values were lower than for OVA with 10:1 being the optimal APC to T cell ratio, but this gave only 2 U/ml at best (EBOpLPP GAD), with positive control wells (BSM transfectants and 33.1 hybridoma cells at a ratio of 5:1 with 6μM of the $GAD_{273-285}$ peptide) giving IL-2 values from 9-33 U/ml. All three GAD transfected cell lines gave IL-2 concentrations above background, but in this case EBOpLPP GAD was functioning the best.

One hypothesis as to the poor IL-2 response for both sets of BSM tranfected cell lines was that the 33.1 and 47.28 hybridomas were made by injecting mice with the peptide. In binding studies, $GAD_{273-285}$ was found to bind very efficiently to DR*0401 (83), and therefore it was used to prime DR4 chimeric mice to make hybridomas. This was also the case for the 47.28 hybridoma -- DQ8 transgenic mice were primed with the $OVA_{323-339}$ peptide following binding studies. To investigate the possibility that the BSM transfected cells were not triggering the T cell hybridomas to capacity because the epitopes in question were not dominant or primary, DQ8 NOD knockout transgenic mice were inoculated with whole OVA and the *in vitro* recall of T cells isolated from draining lymph nodes was analyzed (figure 5.8). BSM cells transfected with the 3 OVA constructs were used as APCs, with cells transfected with the empty vector construct serving as the negative control. IL-2 levels were below 1U/ml; however, in the control wells where $OVA_{323-339}$ peptide was added, the IL-2 values were only 1.2 U/ml at best. This is not unexpected, as in this situation the entire OVA antigen is processed *in vivo* to generate multiple epitopes so that T cells recognizing the 323-339 epitope would occur at a lower frequency.

At this junction the question arose as to whether secondary costimulation necessary for T cell activation was lacking in this system due to a xenogeneic barrier. B7 on APCs has been shown to deliver the costimulatory signal or 'signal two' to the T cells through their CD28 receptor. In an exhaustive literature search no formal evidence of human B7 /

mouse CD28 interactions could be found; however, several references where mouse APCs were able stimulate human T cells were found (176,177). It can not be assumed that the reciprocal of this interaction would work even though the similarities between the human and mouse sequences are striking (178). Another aspect to consider is the necessity of signal two in the first place. Transfection of class II genes into non-antigen presenting cell lines cell has become common. For example, class II molecules have been transfected into murine fibroblast and thymoma (BW 5147) cell lines, neither having endogenous B7, but both were able to stimulate T cells (120,179).

Lastly, to see if any protein expression could be detected in the BSM transfectants, Western blots of lysates of BSM transfected cells were completed (figure 5.9). At the number of cells used ($1x10^6$) no detectable GAD or OVA was present in the transfected BSM cells. The fact that the EBOpLPP transfectants were not positive was a bit puzzling as EBOpLPP expressing the core antigen of hepatitis B virus has been analyzed by Western blot (172); however, the number of cells used in order to get a positive result was not given. Both the controls, commercially prepared OVA and recombinant GAD, were positive. In the GAD positive control a second smaller band just below 45 kDa was seen. This was the result of protein arising from internal translation initiation, and it is seen routinely seen on recombinant GAD preparations. The shorter GAD band is seen because the GAD-6 monoclonal antibody used to blot recognizes an epitope on the C terminus of the GAD molecule.

## CONCLUSIONS

It can be concluded from these data that the pDLEBNA OVA construct was working (as the background concentrations of IL-2 were considerably lower) albeit the concentration of IL-2 produced by T hybridoma cells stimulated with the BSM transfectants was low. Given the complexity of the class II pathway and the size of the OVA (approximately 45

kDa) it seems reasonable that the IL-2 concentrations produced in such a system would not be very high as there would be a number different OVA peptides processed and expressed on the class II molecules. The GAD constructs did not seem to function as well as the OVA. Possible explanations are that GAD has been observed to be toxic in eukaryotic cell lines (John Elliott personal communication). Guilhot et al observed that Protein X from hepatitis B virus could not be stably expressed in EBV cells; even though the DNA was still present no mRNA was transcribed (172). Given the GAD result it is possible that something similar has occurred in pDLEBNA GAD. The vector was obviously still present as the cells continued to thrive under hygromycin B selection; however, perhaps there was genetic rearrangement affecting the GAD gene. To further illuminate this problem northern blots of cell lysates could be done in order to determine if GAD and OVA mRNA was transcribed.

The Ii signal sequence did not seem to elevate the amount of peptide expressed in the class II on the cell surface. There are several possible explanations for this observation. First, perhaps Ii is not necessary; peptides will find their way into the class II compartment without the aid of a signal sequence. This seems an unlikely explanation as there are a growing number of reports in the literature of increased expression of peptides on the class II following fusion with Ii (170,171). A more likely explanation is that the endogenous signal sequences of GAD and OVA interfered with the Ii targeting sequence. The GAD signal sequence is located within the first 45 amino acids (180,181) and the OVA is internal between aa 229-276 (182).

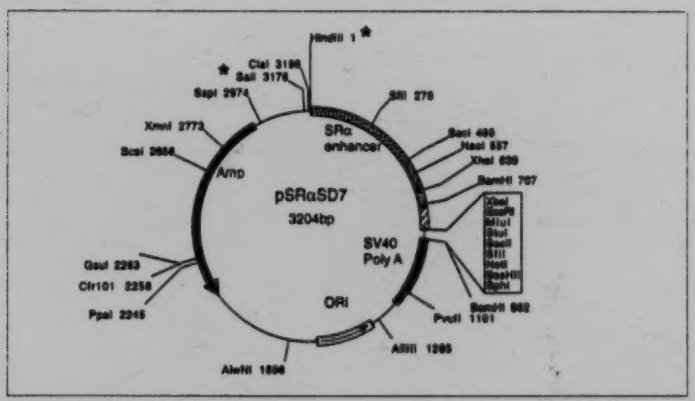
There are several recommendations in order to optimize this system. First, in order to increase protein expression different promoters (e.g. CMV enhancer promoter) could be cassetted into the vector. Another alternative is to continue to use the SRα promoter and transfect in a plasmid encoding the large T antigen (183). This would amplify vector copy number (drives SV40 origin), thereby increasing the amount of protein translated. Other

considerations are the orientation of the promoters. J.M. Young evaluated the amount of IFN produced by several promoters in different orientations (184). It was found IFN was made only when transcription was directed away from *ori* P (e.g. EBNA → *ori* P IFN → ← hph **NOT** EBNA → oriP ← IFN ← hph). This closely resembles the orientation of pDLEBNA (e.g. EBNA → *ori* P ← hph ← GAD) so it is possible that hph transcription across *ori* P inhibits replication -- possibly by preventing binding of EBNA-1. Since the vector remains circular in the nucleus of the cell, promoter occlusion, whereby transcription from one promoter preempts transcription from a second nearby promoter (185) due to physical constraints, is possible. This idea has not been investigated in *ori* P EBNA systems but remains conceivable. Lastly, my vector relies on transcription of the *ori* P from the EBNA-1 and there are reports that this may affect vector replication (184).

The strategy of using EBV based vectors to endogenously synthesize autoantigens and have the peptides associated on the cell surface in class II molecules remains attractive and apparently feasible given my results. Expression was low; however, with some of the simple cloning revisions mentioned above, this system may become useful in the mapping of diabetes autoantigens.

**Figure 5.0.** Assembly of pDLEBNA. Firstly SRαSD7 was cut with the restriction enzymes HindIII and SalI - highlighted with astericks (**a**). The hph PCR product was force cloned into pHEF1αBSD7 (**b**). The strategy is shown below the vector; the SpeI and MfeI sites were incorporated on the ends of the hph during PCR and are compatible with the XbaI and EcoRI sites of the polylinker (highlighted with number signs). The pHEF1αBSD7 was then digested with SalI and ClaI (highlighted with astericks) (**c**). Finally the EBNAoriP was force cloned into pBluescript using the BamHI and ClaI sites (number signs) . Two linkers were added to remove these sites and add new sites; as depicted below the vector (vector sequences are in lower case and linker sequence in upper case). Finally the EBNAoriP portion was removed from pBluescript using ClaI and HindII. The EBNAoriP (ClaI/HindIII), pHEF1αBSD7-hph (SalI/ClaI), and SRαSD7 (HindIII, SalI) were ligated together to create pDLEBNA.

a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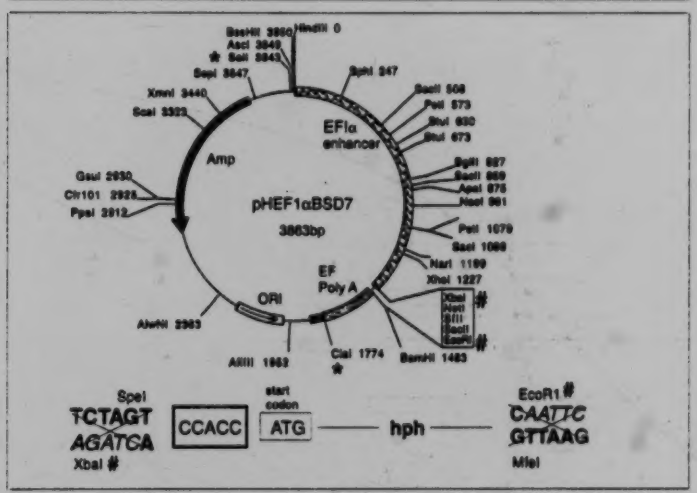
3204bp
SRα enhancer
SV40 Poly A
Amp
ORI

b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EFIα enhancer
EF Poly A
Amp
ORI
SpeI
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hph
EcoR1 #
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MfeI

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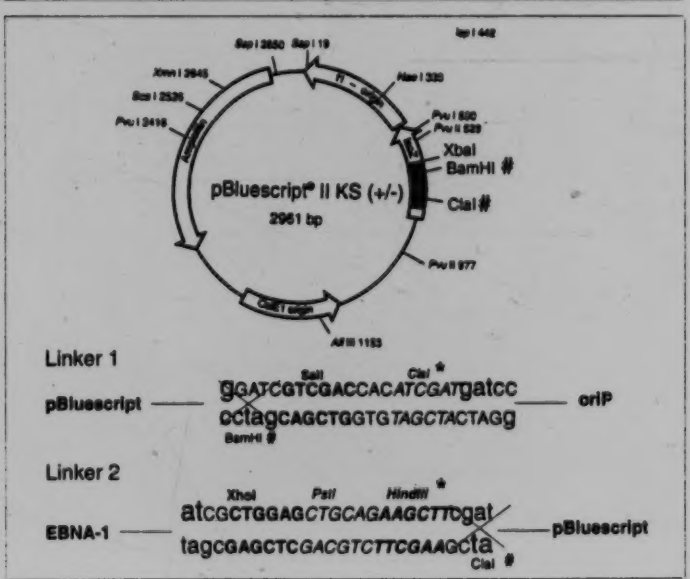
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BamHI #
ClaI #
Linker 1
pBluescript
SalI
ClaI
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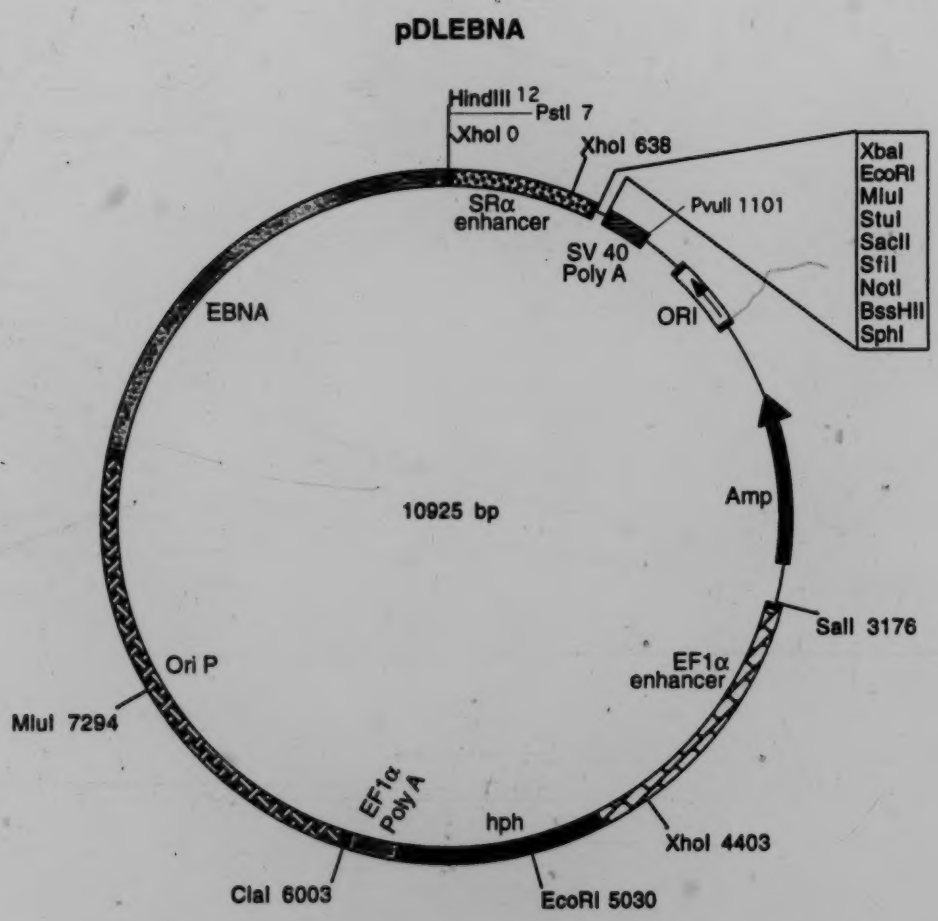



**Figure 5.1**. Map of the pDLEBNA construct. The construct was assembled as described in the Results and Discussion. The SRαSD7, EF1αhph, and EBNAOriP segments were joined using the restriction enzymes HindIII, ClaI, & SalI. Genes were cloned into the plasmid using the XbaI/NotI sites in the polylinker.

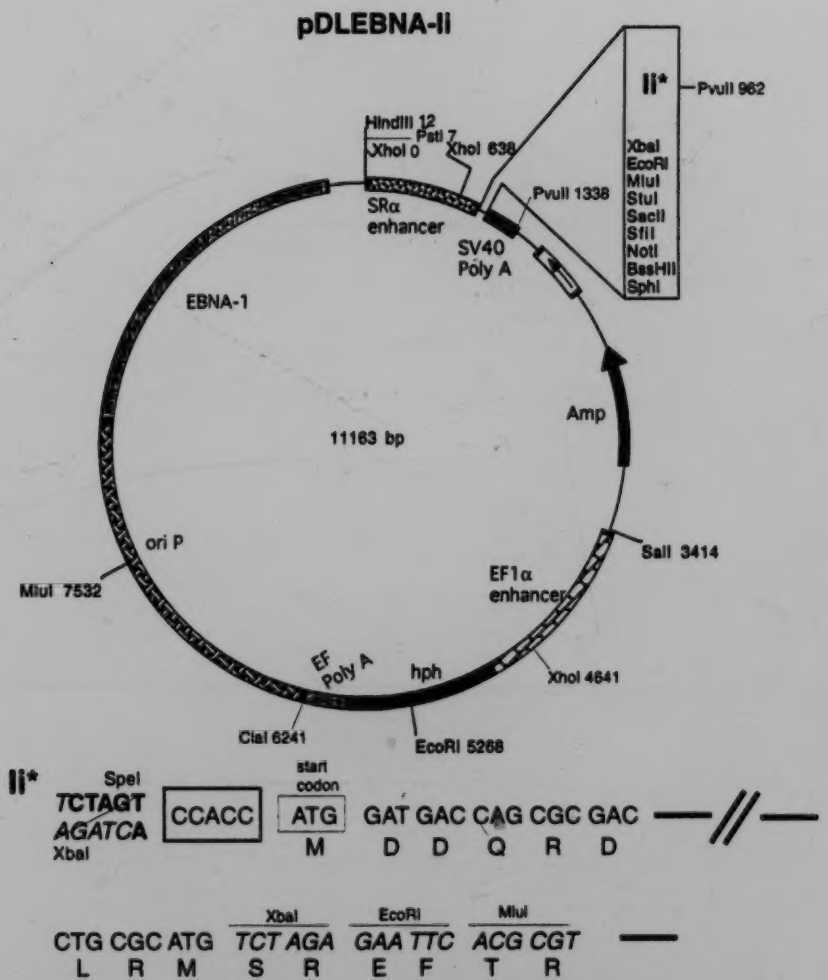
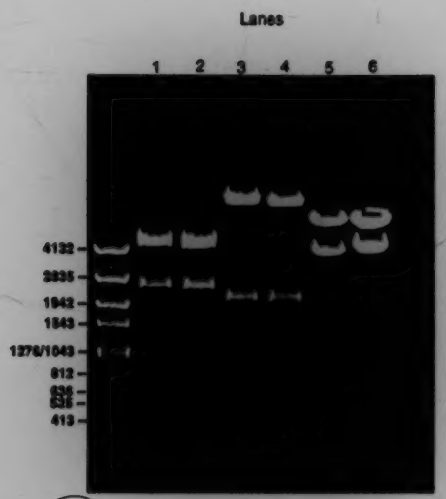



**Figure 5.2.** Map of the pDLEBNA-Ii construct. The first 237bp of the human invariant chain were cloned into the vector pDLEBNA as described in the Results and Discussion. The expression of genes using this plasmid resulted in a fusion protein with the invariant chain signal sequence on the N terminus. The amino acids that created the bridge between the two proteins were encoded by the restriction sites XbaI, EcoRI and Mlu I. The Ii fragment included a SpeI site at the 5' end, which was destroyed when it was ligated to the XbaI site present in the original pDLEBNA vector. There is also a Kozak consensus sequence (heavy box) found on the 5' end of the Ii fragment. Genes were cloned into pDLEBNA-Ii using the 'new' XbaI site supplied at the 3' end of the Ii fragment and NotI from the original polylinker.




| Construct | Restriction enzyme fragment size (bp) | | |
|---|---|---|---|
| | MluI/EcoRI | PvuII/SalI | XhoI |
| pDLEBNA | 4431<br>4230 lane 1<br>2264 | 8850<br>2075 lane 3 | 6529<br>3765 lane 5<br>631 |
| pDLEBNA-Ii | 4681<br>4218 lane 2<br>2264 | 8712<br>2075 lane 4<br>376 | 6529<br>4003 lane 6<br>631 |

**Figure 5.3.** Restriction fragment analysis of pDLEBNA and pDLEBNA-Ii. Constructs were mapped using the sites depicted in Figures 5.1 and 5.2. Expected fragments lengths (from the table) correlate with those seen on the agarose gel. The two longer MluI/EcoRI fragments (seen as a single bright band in lanes 1 and 2) overlap on this gel, but could clearly be seen on lower percentage agarose gels (data not shown). pDLEBNA-Ii has an extra PvuII site located within the invariant chain sequence and this resuleds in the additional faint band at 376bp seen in lane 4.

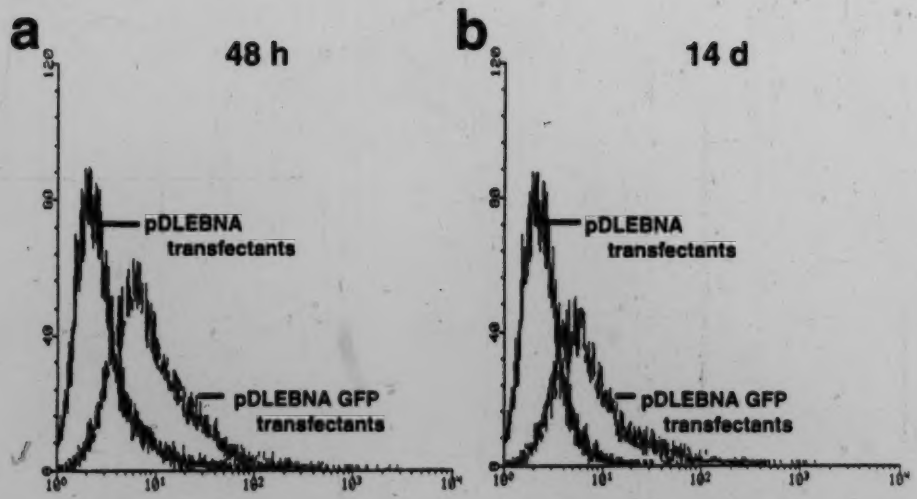



**Figure 5.4.** Testing the function of pDLEBNA using GFP as a reporter. BSM cells were electroporated with either the pDLEBNA or pDLEBNA GFP plasmids as described in the Materials and Methods. The transfectants were placed under hygromycin B selection (200µg/ml) and the resulting cell populations were monitored for GFP expression using FACS analysis at 48h (**a**) and 14d (**b**) post electroporation.

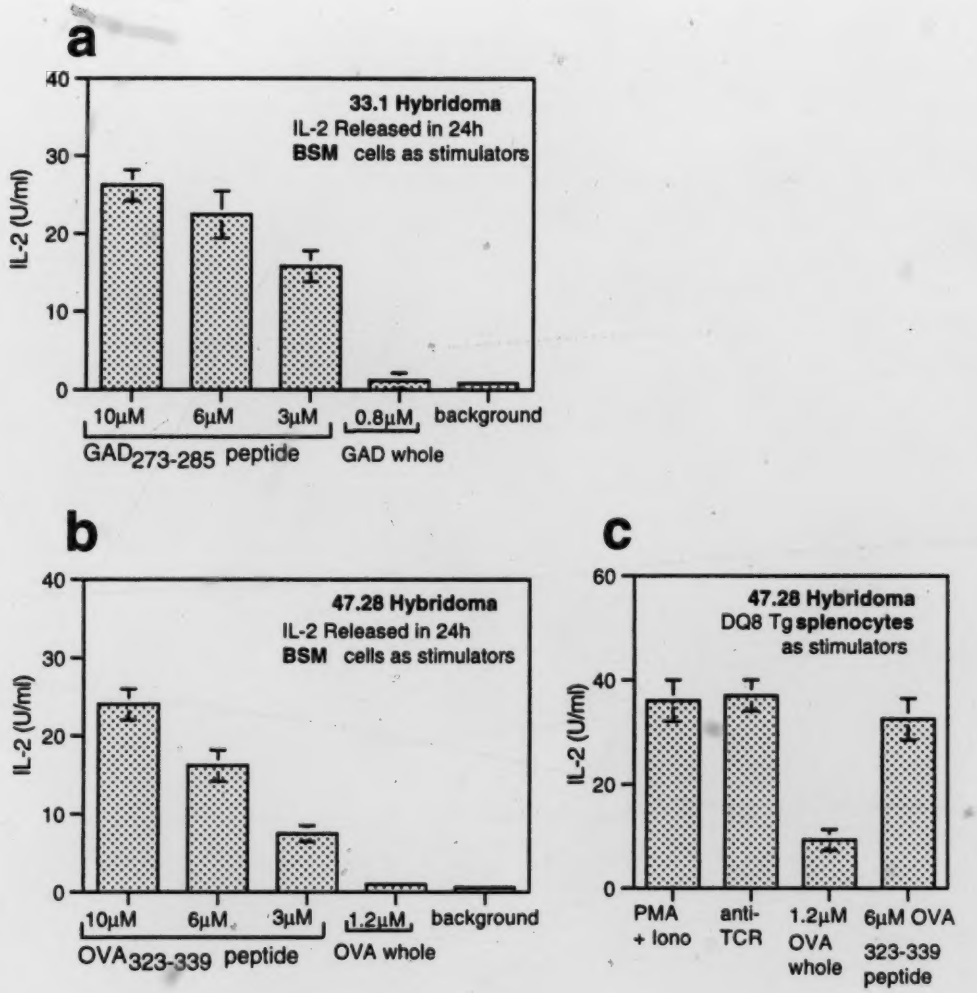
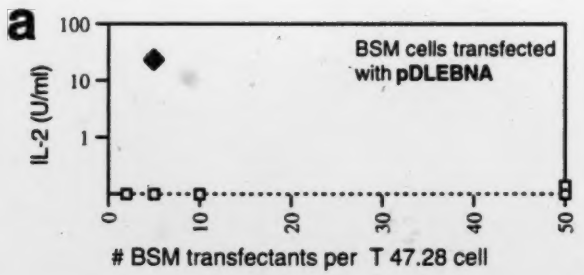
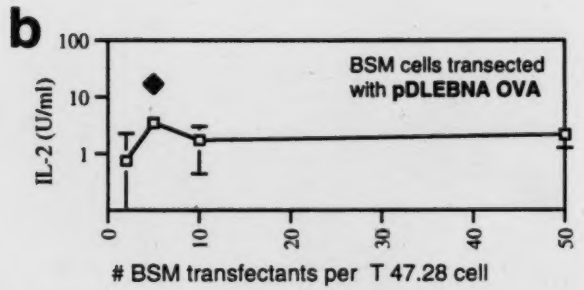
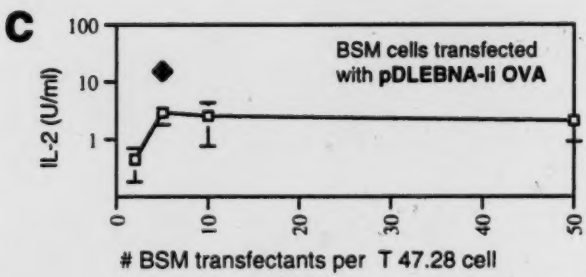



**Figure 5.5.** *In vitro* stimulation of OVA and GAD specific T cell hybridomas. Specificity of the 33.1 hybridoma (sees $GAD_{273-275}$ on DR4) (Panel **a**) and the 47.28 hybridoma (sees $OVA_{323-339}$ on DQ8) (Panel **b**) were tested using BSM typing cells (**a&b**) or $IA_{\beta}^{-/-}$ / $DQ8^{+/+}$ splenocytes (**c**) as APCs and the appropriate peptide or whole protein. IL-2 released in 24 h was measured by ELISA. Background concentrations of IL-2 released were the same in panels b and c. Note that the EBV transformed BSM cells express both DR4 and DQ8.

**Figure 5.6.** Capacity of transfected BSM cells to stimulate the OVA specific T cell hybridoma. pDLEBNA (**a**), pDLEBNA OVA (**b**), pDLEBNA-Ii OVA (**c**), EBOpLPP OVA (**d**) were electroporated into BSM typing cells and placed under hygromycin selection. The resistant cell populations were used to stimulate the 47.28 hybridoma cell specific for the $OVA_{323-339}$ peptide. The empty vector, pDLEBNA, served as the negative control (**a**). Open squares indicate the concentration of IL-2 released in 24h; a closed diamond indicates the concentration of IL-2 released when 6μM of the $OVA_{323-339}$ peptide was added into the assay using 5 BSM cells per T cell. The number of BSM cells per T hybridoma cell was varied (2,5,10,50:1) since it was unclear which ratio would give optimal stimulation.


a
100
10
1
IL-2 (U/ml)
BSM cells transfected
with pDLEBNA
0
10
20
30
40
50
# BSM transfectants per T 47.28 cell


b
100
10
1
IL-2 (U/ml)
BSM cells transected
with pDLEBNA OVA
0
10
20
30
40
50
# BSM transfectants per T 47.28 cell


c
100
10
1
IL-2 (U/ml)
BSM cells transfected
with pDLEBNA-li OVA
0
10
20
30
40
50
# BSM transfectants per T 47.28 cell

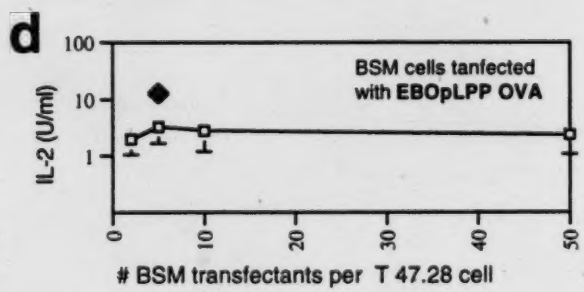
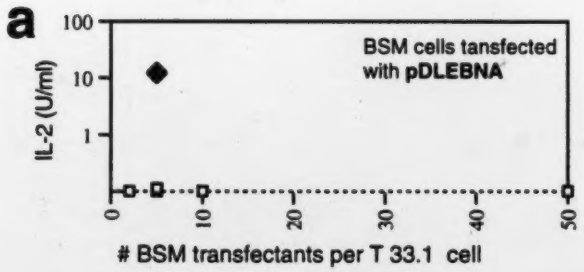
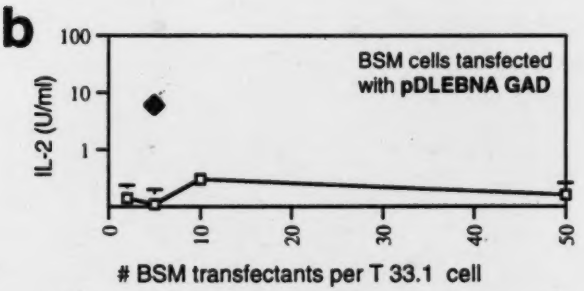
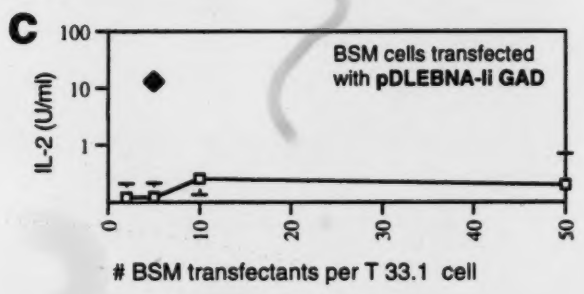
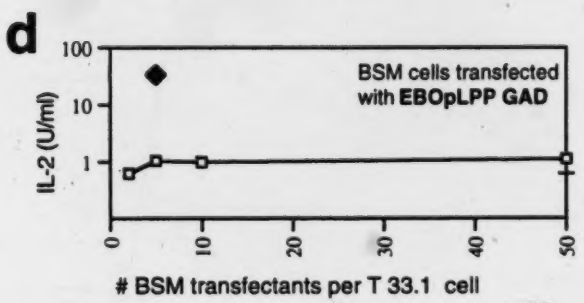

d
100
10
1
IL-2 (U/ml)
BSM cells tanfected
with EBOpLPP OVA
0
10
20
30
40
50
# BSM transfectants per T 47.28 cell

**Figure 5.7.** Capacity of transfected BSM cells to stimulate the GAD specific T cell hybridoma. pDLEBNA (**a**), pDLEBNA GAD (**b**), pDLEBNA-Ii GAD(**c**), EBOpLPP GAD (**d**) were electroporated into BSM typing cells and placed under hygromycin selection. The resistant cell populations were used to stimulate the 33.1 hybridoma cell specific for the $GAD_{273-285}$ peptide. The empty vector, pDLEBNA, served as the negative control (**a**). Open squares indicate the concentration of IL-2 released in 24h; a closed diamond indicates the concentration of IL-2 released when 6μM of the $GAD_{273-285}$ peptide was added into the assay using 5 BSM cells per T cell. The number of BSM cells per T hybridoma cell was varied (2,5,10,50:1) since it was unclear which ratio would give optimal stimulation.


a
100
10
1
IL-2 (U/ml)
BSM cells tansfected
with pDLEBNA
0
10
20
30
40
50
# BSM transfectants per T 33.1 cell


b
100
10
1
IL-2 (U/ml)
BSM cells tansfected
with pDLEBNA GAD
0
10
20
30
40
50
# BSM transfectants per T 33.1 cell


c
100
10
1
IL-2 (U/ml)
BSM cells transfected
with pDLEBNA-Ii GAD
0
10
20
30
40
50
# BSM transfectants per T 33.1 cell


d
100
10
1
IL-2 (U/ml)
BSM cells transfected
with EBOpLPP GAD
0
10
20
30
40
50
# BSM transfectants per T 33.1 cell

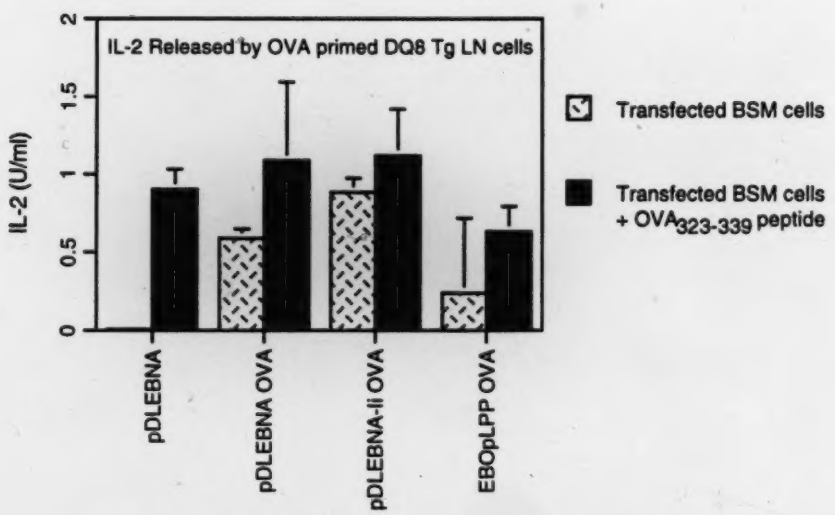



**Figure 5.8.** Responses of DQ8 transgenic, OVA primed T cells to BSM transfectants. BSM cells ($2x10^5$/well) transfected with OVA constructs were used as APCs on T cells ($1x10^5$/well) harvested from OVA/CFA immunized $IA_\beta^{-/-}$ $DQ8^{+/+}$ mice. Cultures (final volume 200 µl) were incubated for 24h and IL-2 concentrations measured by ELISA. The positive control (solid bars) was 6µM of the $OVA_{323-339}$ peptide added into the assay.

a

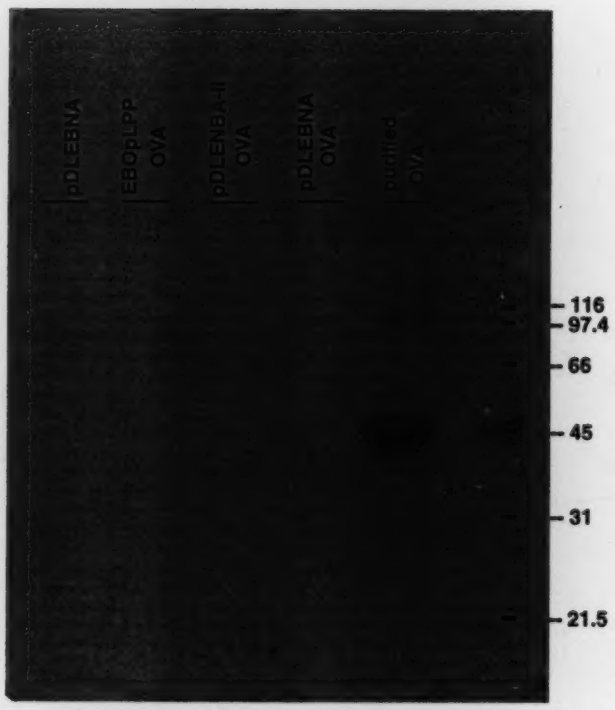
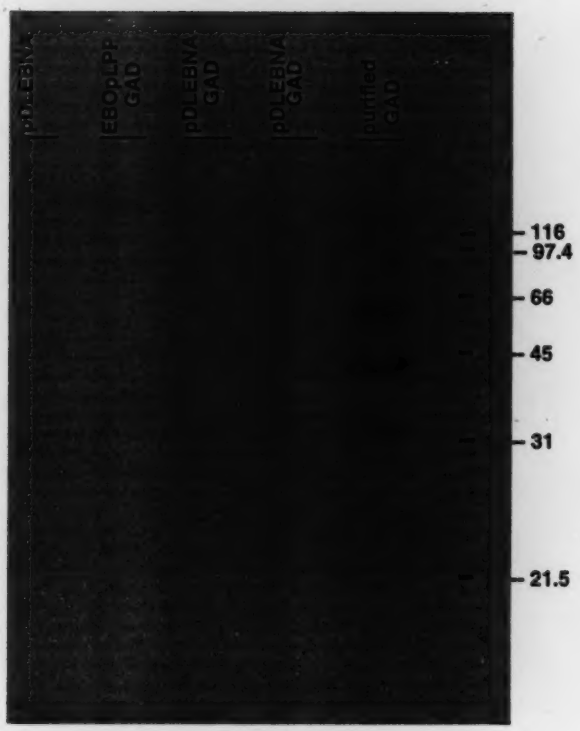



**Figure 5.9**. Western blots of OVA & GAD transfected BSM cells. BSM cells (1x10 ) tranfected with various OVA (**a**) or GAD (**b**) constructs were harvested and lysed as described in the Materials & Methods. The lysates were electrophoresed on a 10% polyacrylamide gel, transfered to a nitrocellulose membrane and incubated with mouse anti-OVA anti-serum (1:10 000) (Panel **a**) or GAD-6 monoclonal antibody (1:20 of hybridoma cell supernatant Panel **b**). Purified proteins(10µg per lane) were used as the positive control. The smaller band seen in the purified GAD lane corresponds to a second internal initiation codon.

**b**


pD.EBNA
EBOV.LPP GAD
pD.EBNA GAD
pD.EBNA GAD
purified GAD
116
97.4
66
45
31
21.5

# CHAPTER VI

## THE STUDY OF TRANS-HETERODIMERS OF $IA_\alpha^b$ WITH $IA_\beta^{g7}$

### INTRODUCTION

Our collaborator, Dr. T. Jevnikar of the University of Western Ontario (London, Ont.), has observed that when his NOD class II knockout mice were back-crossed with NOD/Lt animals, the F1 progeny had the same low incidence of diabetes as the parental knockout mice. In these F1 animals, one chromosome had $IA_\alpha^b$ plus the $IA_\beta^{null}$, and the other contained $IA_\alpha^d$ and $IA_\beta^{g7}$. Therefore the NOD class II molecule was expressed, albeit perhaps at a lower level. Because the original knockout mice are H-$2^b$, by repeatedly back-crossing NOD/Lt with the knockout mice and selecting for $IA^{null}$, the entire MHC region becomes fixed on the b haplotype. Failure of the F1 mice to develop diabetes therefore may be related to the addition of b haplotype class I molecules in these animal, or to the presence of an altered class II MHC molecule. This latter possibility would come about if the $IA_\alpha^b$ molecule formed a trans-heterodimer with the NOD $IA_\beta^{g7}$ molecule. From the literature it was unclear if such trans-heterodimers exist. To answer this question COS-7 cells (SV40 transformed African green monkey kidney cells) and HeLa cells (epitheloid human cervical carcinoma cells) were transiently transfected with combinations of various individual IA $\alpha$ and $\beta$ chains, and the transfectants analyzed by FACScan for surface expression of the relevant class II heterodimers.

Two different antibodies were used in these experiments--10-3.6 (109) and Y-3P (110). Previous studies by other groups have shown that the 10-3.6 antibody stains the beta chains of $IA^k$ as well as $IA^{g7}$ (109,186). The Y-3P antibody most likely stains the $IA_\alpha^b$ chain, since it has been shown to stain $IA\alpha^b/IA_\beta^b$ and $IA_\alpha^b/IA_\beta^k$, but not to stain $IA_\alpha^d/IA_\beta^d$ (186). It is also possible that Y-3P sees a slightly more complex determinant,

possibly over the top of the class II molecule, since it also showed weak staining to cells transfected with $IA_\alpha^k/IA_\beta^b$ and cross reacts with cells expressing $IA_\alpha^k/IA_\beta^k$. (187)

## RESULTS AND DISCUSSION

PCR products were first visualized on an agarose gel to ensure they were the correct size (figure 6.1 a). Approximately the first 250 bp of the $IA_\alpha^b$ and $IA_\beta^b$ clones were sequenced and no mutations were present (data not shown). Before the plasmids were used in the eukaryotic expression systems they were also mapped using restriction enzymes (figure 6.1 b & c). Firstly all of the constructs were digested with XbaI / NotI (NEB) following the manufactures' directions. This separated the IA cDNA from the plasmid backbone to ensure the size was correct. The clones were then digested with different restriction enzymes (figure 6.1 b). Sites were chosen that were found once within the cDNA and once within the plasmid in order to carefully map the clones. Expected sizes were calculated and are listed in the table of figure 6.1 d.

COS-7 cells were transfected with the following combinations of cDNA expression plasmids: 1) 'empty' vector control , 2) $IA_\alpha^d$ / $IA_\beta^{g7}$, 3) $IA_\alpha^b$ / $IA_\beta^k$, 4) $IA_\alpha^b$ / $IA_\beta^{g7}$. Following the 72 hour incubation the cells were removed from the monolayers and stained with either FITC conjugated 10-3.6 or Y-3P and analyzed by FACS. Figure 6.2 shows FACS profiles that were representative of results obtained from three separate experiments. The COS-7 cells were difficult to analyze by FACS due to their large size and tendency to clump together even after being forced through a nylon mesh . These results suggested that the $IA_\alpha^b$ and $IA_\beta^{g7}$ combination was not staining on the surface of the COS cells (figure 462 g&h) but since the data was of relatively poor quality because of the difficulty in FACS analysis of COS cells, the experiment was repeated using HeLa cells.

HeLa cells were transfected by electroporation with the following combinations of cDNA expression plasmids: 1) 'empty' vector control , 2) $IA_\alpha^d$ / $IA_\beta^{g7}$, 3) $IA_\alpha^b$ / $IA_\beta^k$,

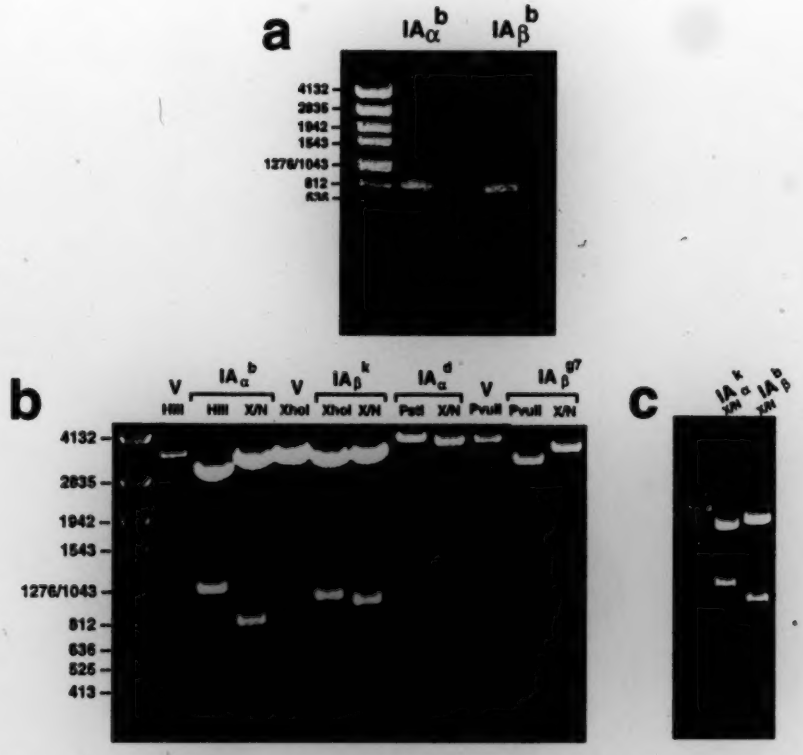
4) $IA_{\alpha}^{b}$ / $IA_{\beta}^{g7}$, 5) $IA_{\alpha}^{b}$ / $IA_{\beta}^{b}$, 6) $IA_{\alpha}^{k}$ / $IA_{\beta}^{k}$. After 72 hours in culture the cells were removed from the monolayers, stained with either 10-3.6 or Y-3P , and then analyzed by FACScan. Representative FACS profiles for these experiments are shown in figure 6.3. These profiles confirm that all of the cDNAs were pairing as expected. IA $_{\alpha}^{b}$ / $IA_{\beta}^{k}$ stained positively with Y-3P and 10-3.6; $IA^{NOD}$ stained with 10-3.6 but not Y-3P. Cross reactive staining was seen with $IA_{\alpha}^{k}$ / $IA_{\beta}^{k}$ when stained with Y-3P and also with $IA_{\alpha}^{b}$ / $IA_{\beta}^{b}$ when stained with 10-3.6 as expected (186). The profiles also indicated that $IA_{\alpha}^{b}$ and $IA_{\beta}^{g7}$ were not present on the cell surface as a heterodimer. The limitations of the experiments were that neither of the eukaryotic cells used have a functional invariant chain. This could of played a role in the transport of the $IA_{\alpha}^{b}$ and $IA_{\beta}^{g7}$ to the cell surface; however, it is unlikely that this was significant as all other combinations of IA chains paired and were expressed on the cell surface as expected. There are reports in the literature of HeLa and COS cells transfected with class II that exhibited no difference in cell surface expression when Ii was introduced (22,188,189).

## CONCLUSIONS

Three conclusions can be drawn from this data. Firstly, the data indicates that both the $IA_{\alpha}^{b}$ and $IA_{\beta}^{g7}$ plasmids were functional, since they induced surface heterodimers when co-transfected with complementary chains of haplotypes that they are known to pair with (i.e. $IA_{\alpha}^{b}$ pairs with $IA_{\beta}^{k}$ and $IA_{\beta}^{b}$; $IA_{\alpha}^{d}$ pairs with $IA_{\beta}^{g7}$). Secondly, the antibodies stained class II combinations as expected as 10-3.6 stained $IA^{k}$, $IA_{\alpha}^{b}$ with $IA_{\beta}^{k}$, as well as $IA^{NOD}$, whereas Y-3P stained $IA^{b}$ and $IA_{\alpha}^{b}$ with $IA_{\beta}^{k}$, but not $IA^{NOD}$. Thirdly, $IA_{\alpha}^{b}$ does not form a surface heterodimer with $IA_{\beta}^{g7}$.

These results appeared to exclude the possibility that the failure of the F1 mice to develop diabetes was related to the presence of an altered class II MHC molecule consisting of a trans-heterodimer between $IA\alpha^{b}$ and $IA_{\beta}^{g7}$ but rather emphasizes that addition of the $K^{b}$ class I molecules in these animals is likely responsible for protection

from disease. Alternately, we cannot rule out the possibility that lower levels of class II, and/or other MHC complex genes of the b haplotype might be responsible for these effects.




d

| Construct | Expected restriction fragment sizes (bp) | |
|---|---|---|
| | Unique site | XbaI/NotI (X/N) |
| $IA_{\alpha}^{b}$ | 2971 (HindIII)<br>1088 | 3204<br>855 |
| $IA_{\beta}^{k}$ | 2883 (XhoI)<br>1003 | 2920<br>966 |
| $IA_{\alpha}^{d}$ | 3568 (PstI)<br>901 | 3204<br>1265 |
| $IA_{\beta}^{g7}$ | 2599 (PvuII)<br>1865 | 3204<br>1260 |
| $IA_{\beta}^{b}$ | | 3204<br>844 |
| $IA_{\alpha}^{k}$ | | 2920<br>982 |

**Figure 6.1.** Creation and verification of IA clones. RT-PCR of $IA_{\alpha}^{b}$ / $IA_{\beta}^{b}$ coding sequences from spleen RNA as described in the Materials and Methods (**a**). Restriction digests of constructs using enzymes expected to cut once in the vector and once in the IA clone (**b**). Expected fragment lengths are given in (**d**). IA clones were also cut with XbaI/NotI to drop out the entire cDNA insert in order to confirm size (**b&c**) as summerized in (**d**). Empty vector SRαSD7 (labelled V on gel **b**) was cut with Hind III, XhoI, and PvuII and the plasmid was linearized as expected.

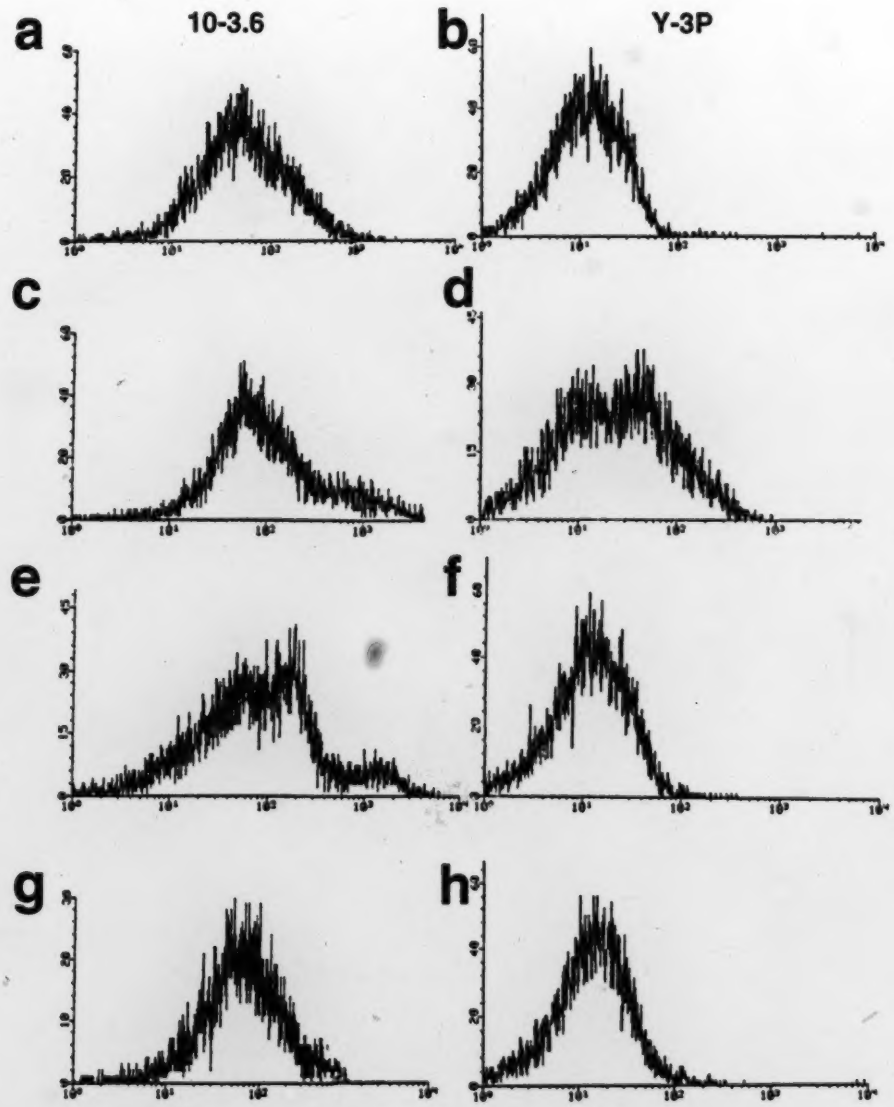
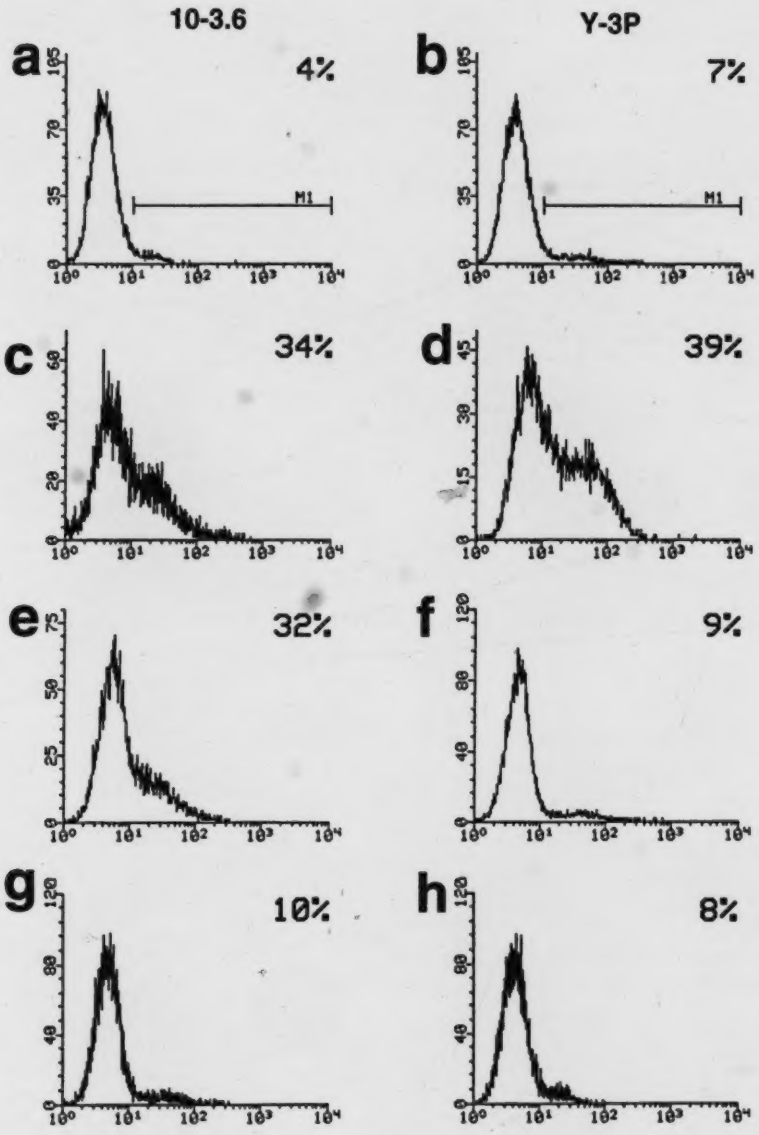



**Figure 6.2.** FACS profiles of COS cells transiently transfected with combinations of cDNAs for various IA chains, then stained with FITC conjugated antibodies 10-3-6 (**a,c,e,g**) or Y-3P (**b,d,f,h**). Cells were transfected with the following plasmids: **a&b** - 'empty' plasmid control; **c&d** - $IA_{\alpha}^{b}$ /$IA_{\beta}^{k}$; **e&f** - $IA_{\alpha}^{d}$ /$IA_{\beta}^{g7}$; **g&h** - $IA_{\alpha}^{b}$ /$IA_{\beta}^{g7}$. Panels **a&b** -- negative controls; panels **c,d,e&f** -- positive controls; and panels **g&h** -- experimental.


10-3.6
Y-3P
a
4%
b
7%
M1
M1
c
34%
d
39%
e
32%
f
9%
g
10%
h
8%

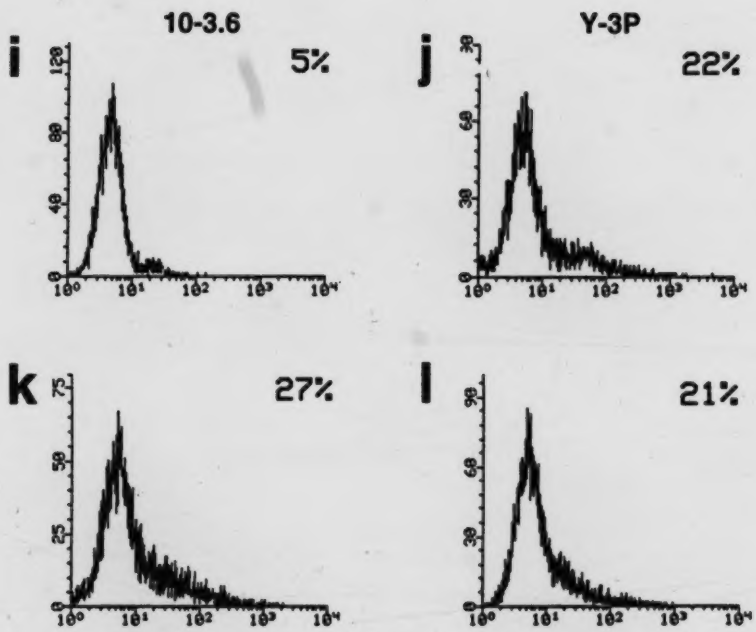



**Figure 6.3.** FACS profiles of HeLa cells transiently transfected with combinations of cDNAs for various IA chains, then stained with FITC conjugated antibodies 10-3.6 (**a,c,e,g,i,k**) or Y-3P (**b,d,f,h,j,l**). Cells were transfected with the following plasmids: a&b - 'empty' plasmid control; **c&d** - $IA_\alpha^b$ /$IA_\beta^k$; **e&f** - $IA_\alpha^d$ /$IA_\beta^{g7}$; **g&h** - $IA_\alpha^b$ /$IA_\beta^{g7}$; **i&j** - $IA_\alpha^b$/$IA_\beta^b$; **k&l** - $IA_\alpha^k$/$IA_\beta^k$. Portion of the cells within the marker M1 are expressed as percentages for each FACS plot. Panels **a&b** - negative control; panels **c,d,e,f,i,j,k,&l** - positive controls; panels **g&h** - experimental.

# CHAPTER VII

## GENERAL CONCLUSIONS

The goal of this thesis was to pave the way for the mapping of T cell epitopes from diabetic autoantigens. The rationale was to create an animal model that expressed the human genetic susceptibility gene, HLA-DQ8. In the beginning chimeric human / mouse transgenic animals were constructed. Due to a failure of the cDNA expression plasmid, these animals did not express functional human MHC class II. As a second approach to map T cell epitopes, in collaboration with Dr. L. Wicker from Merck Research Laboratories and Dr. T. Jevnikar from the University of Western Ontario, we bred NOD mice transgenic for the human DQ8 with NOD class II knockout mice. This was to remove all endogenous murine class II in order to ensure all immune responses generated would be due to the human class II. Preliminary characterization of these animals showed that part of their immune system has been restored upon the addition of class II, for example, the peripheral CD4 T cell count increased from approximately 1% to approximately 11%. This is about half of the normal number of CD4 cells but seems to be sufficient in the generation of an immune response to antigens such as OVA. Immune responses to putative diabetes autoantigens such as GAD and proinsulin were not as impressive as the OVA; however, with small changes to protocols (e.g. optimizing stimulation conditions) hopefully this can be rectified. Interestingly, the DQ8 NOD knockout mice may be a useful tool in the clarification of superantigen binding to DQ8. All three superantigens studied were able to stimulate T cells from a DQ8 NOD knockout mouse and there are other superantigens with undefined HLA isotype preferences (190).

In an attempt to develop new tools to make the mapping of T cell epitopes easier and more cost effective, an EBV based expression vector was created. The hypothesis

was that the cDNA from autoantigens could be expressed in APCs using this vector, and hopefully a small portion of the protein would make its way into the class II compartment and onto the cell surface bound to the class II molecules. Subfragments of large proteins could be expressed; theoretically decreasing the number of overlapping peptides that ultimately would need to be made. In its present form, the vector is of little value as the protein expression is too low but with minor changes to the vector structure it could still prove to be a useful tool in the future. In collaboration with Dr. Jevnikar a trans-heterodimer experiment was completed in order to determine if the low incidence of diabetes in NOD knockout mice backcrossed onto NOD was due to a trans-pairing of $IA_{\alpha}^{b}$ with $IA_{\beta}^{g7}$. Through transient expression of these cDNAs in two eukaryotic cell lines it was found that these molecules do not pair and the low incidence of diabetes must be due to other diabetic susceptibility genes (e.g. class I).

The goal of this project, to develop a mechanism to map T cell epitopes in diabetes autoantigens, has been accomplished. DQ8 NOD knockout mice can be injected with antigens and the immune response analyzed. Hopefully these mice will be a valuable tool in the furthering of our understanding of the autoimmune mechanisms of diabetes.

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# APPENDIX I

## CREATION OF A MURINE FIBROBLAST CELL LINE EXPRESSING $IA^{NOD}$

### INTRODUCTION

In this project we created an NOD antigen presenting cell line in order to characterize NOD T cell hybridomas created by Dr. B. Singh and Dr. J. Madrenas (University of Western Ontario). DAP.3, a mouse fibroblast cell line (190) that does not express endogenous class II was chosen. $IA_\alpha^{NOD}$ and $IA_\beta^{NOD}$ cDNAs were transfected into these cells together with the neomycin resistance gene, and stable transfectants selected for using the antibiotic geneticin (G-418).

### MATERIALS AND METHODS

**1. Cell lines, plasmids & antibodies**

The DAP.3 were kindly supplied by Q. Madrenas. They were maintained in complete DME [DME supplemented with 10% FCS and 50μM 2–ME]. The 10-3.6 hybridoma (ATCC TIB 92) was grown in bulk cultures, antibodies purified by affinity chromatography over protein A sepharose, and conjugated to FITC using standard methods (109). The $IA_\alpha^{NOD}$ and $IA_\beta^{NOD}$ were previously described in the Materials and Methods p23. The neomycin selection gene (pSSDneo) was kindly provided by D. Denney. All cDNAs were purified on CsCl/Ethidium Bromide gradients as described in the Materials and Methods p 35.

**2. Electroporation of DAP.3 cells**

$IA_\alpha^{NOD}$, $IA_\beta^{NOD}$ (200μg each), and pSSDneo (40μg) were linearized overnight

using the restriction enzyme Sal I. The digested DNA was mixed together, precipitated in 95% ethanol /0.3M sodium acetate, and resuspended in 400µl HBSE [20mM Hepes pH 7.0, 0.75mM $NaHPO_4/NaH_2PO_4$ pH 7.0, 138mM NaCl, 5mM KCl, 5.5mM Dextrose]. For electroporation confluent DAP.3 cells were removed from the plate using Trypsin EDTA and washed once with HBSE. The cells ($1x10^7$) were resuspended in 400µl HBSE and electroporated in a 0.4 cm cuvette with a Gene Pulser Electroporator (BioRad, Richmond, CA) set at 260V and 960µF. The cells were plated out onto five 150 x 15mm plates (Falcon 3025) in 25ml complete DME media containing 100µg/ml gentamicin (Gibco 11811-031). At 24 hours the media was removed and replace with 25ml fresh media also containing G-418 (400µg/ml).

### 3. Class II cell surface staining and fluorescence activated cell sorting

Fourteen days later the cells were stained for surface expression of $IA^{NOD}$ (for details see Materials and Methods p??). The cells were analyzed by Dorothy Rutkowski on a Coulter Cytometry Epics Elite, Coulter Canada and 10 000 of the brightest staining cells were collected. This was completed on three occasions after the cells had reached a sufficient density.

## RESULTS AND DISCUSSION

Because of the G418 selection only the cell populations where the neomycin resistance gene integrated into the chromosome would successfully grow. This aided in the determination of the IA status as all cells that were untransfected were killed. With each round of FACS analysis for $IA^{NOD}$ the cell population became more homogeneous as shown in figure A.1. These plots show the cell profile on the first sort after two weeks in selection (figure A.1 a) and on the last sort after six weeks (figure A.1 b). With this method of selecting the only population of cells that would be present would be cells that

contained both the a and b chains as both are necessary for class II cell surface expression. At this point the cells were removed from selection and frozen.

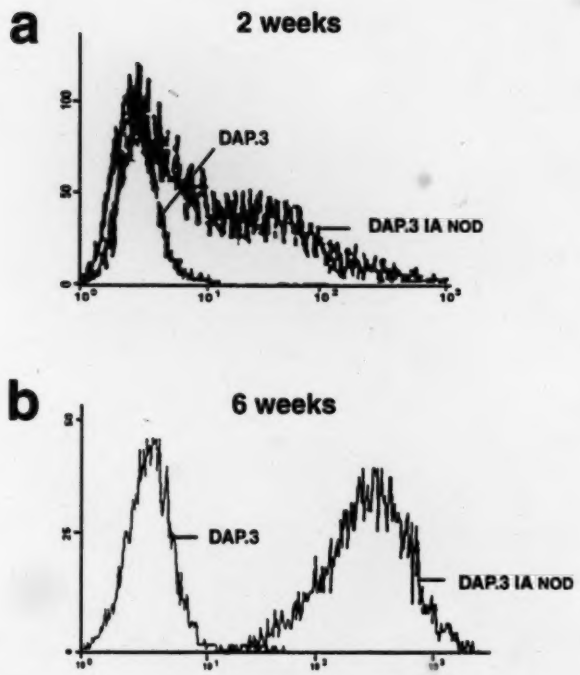


**Figure A.1**. FACS analysis of DAP.3 stable transfectants. DAP.3 cells were electroporated with 200μg of each IA NOD chain and selected for using G418. Cells expressing functional IA NOD on their surface were selected for by staining with FITC conjugated 10-3.6 and collecting the brightest staining population. Staining was done at (**a**) two weeks and (**b**) six weeks post-electroporation. FACS profiles are overlays of untransfected DAP.3 cells and DAP.3 IA NOD cells.